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The Nutritional Requirements of *Haemophilus pertussis*

BY W. H. H. JEBB AND A. H. TOMLINSON

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SUMMARY: Cohen & Wheeler medium prepared from casein hydrolysate previously treated with charcoal supported the growth of small inocula of *Haemophilus pertussis* (1–10 organisms/ml.).

Almost as good growth was obtained from small inocula in medium in which the casein hydrolysate was replaced by a balanced mixture of 17 amino acids and which contained a mixture of growth factors.

Medium containing either cystine and glutamic acid or cystine and aspartic acid as the only amino acids also supported the growth of small inocula; the rate of growth, however, was low.

Nicotinic acid proved to be an essential growth factor for all strains tested. Guanine, hypoxanthine and xanthine enhanced growth although they were not essential. Biotin and haemin, also not essential, enhanced growth during the early phase; this was especially evident with freshly isolated strains.

Hornibrook (1939, 1940) originally showed that phase I *Haemophilus pertussis* was able to grow in a liquid medium consisting of salts, starch and casein hydrolysate or a mixture of amino acids and that nicotinic acid was necessary for growth. Later workers (Farrell & Taylor, 1945; Ungar, James, Muggleton, Pegler & Tomich, 1950; Watanabe, Nishimura & Tomita, 1952; Imamura, 1952) confirmed the requirement for nicotinic acid. Farrell & Taylor and Watanabe *et al.* found thiamine to be essential for some strains, and the latter workers reported stimulation of growth by riboflavin, *p*-aminobenzoic acid and pyridoxine. Imamura (1952) showed that *H. pertussis* would grow in a medium containing cystine and glutamic acid or proline as the only amino acids.

The large inocula (*c.* 10^6 cells/ml.) used by all these workers were not satisfactory for nutritional studies, since media which are suitable for the growth of large inocula are frequently inadequate for small inocula. This present study was undertaken to determine the requirements for full growth of small inocula.

MATERIALS AND METHODS

Strains. The strains used were isolated in this laboratory; some were kept freeze-dried until required and others tested within 12 days after isolation. Strain 724 was used in a previous study (Jebb & Tomlinson, 1951). Strains in constant use were re-dried by Stamp's (1947) method and a fresh tube opened monthly. Periodic tests showed that the strains always agglutinated to titre with phase I serum.

Preparation of inocula. A 1- or 2-day old culture on Bordet-Gengou medium was inoculated heavily on to the same medium and incubated at 35° for 18 hr.

The growth was washed off with saline, centrifuged, washed twice with saline, resuspended to a known opacity and diluted for use. Counts performed occasionally showed that the usual inoculum contained *c.* 10^3 viable cells.

Conditions of incubation and measurement of growth. In order to keep the surface to volume ratio as high as possible and to enable the optical density to be read directly in the growth vessels, 5 ml. of medium were placed in 6 × $\frac{5}{8}$ in. test tubes bent, at an angle of 45°, 1½ in. from the open end. The tubes were fitted with aluminium caps and incubated horizontally at 35°. In these tubes growth after 90 hr. was 6 times as great as that in vertical test tubes.

Growth was measured turbidimetrically against uninoculated blanks, using a Hilger 'Biochem' absorptiometer with a blue filter (Chance OB 2). The figures given are the means of the extinctions for triplicate tubes. The extinction was proportional to the number of cells up to a reading of 0·85, and a reading of 0·50 was equivalent to 270 μg. dry wt./ml. or *c.* $1{\cdot}3 \times 10^{10}$ cells/ml. (American standard for pertussis vaccine).

Modified Cohen & Wheeler medium (*C.W.*). Cohen & Wheeler medium (1946) was chosen as the control. This medium prepared according to their method was unable to support the growth of inocula smaller than 10^6 cells/ml. When, however, the casein hydrolysate (Difco Casamino Acids, Technical) was adsorbed with charcoal, for the purpose of removing growth factors, it was found that the resulting medium supported the growth of small inocula of the order of 1–10 organisms/ml.—the charcoal, presumably, had removed some growth inhibitor. The following further modifications also gave better and more consistent growth: (i) calcium and copper salts were omitted and the amount of magnesium chloride reduced to one-tenth; (ii) cysteine was replaced by cystine; (iii) the pH was raised to 7·9 (cf. Ungar *et al.* 1950); and (iv) the yeast dialysate was decreased to 1·25 %.

The method finally adopted for preparing the modified C.W. medium was as follows: 20 g. Casamino Acids were dissolved in a litre of distilled water, adjusted to pH 2·4 and adsorbed with 6 g. of Norit charcoal (Harrington) at 100° for 30 min. The mixture was filtered and adjusted to pH 6·8 and adsorbed again with charcoal at 100° for 30 min. The mixture was filtered, and 6 g. charcoal were added and the whole allowed to stand at room temperature for 2 hr. After filtering the volume was made up to 1 l. The solution was stored frozen and made into medium as required: adsorbed casein hydrolysate solution, 500 ml.; KH_2PO_4, 0·5 g.; NaCl, 2·5 g.; $MgCl_2.6H_2O$, 0·04 g.; $FeSO_4.7H_2O$, 0·01 g.; L-cystine, 25 mg.; soluble starch, 1·5 g.; water to 800 ml. The casein hydrolysate, water and starch were boiled for 7 min. After cooling, the salts and cystine were added, the volume made up to 800 ml. and the pH adjusted to 7·9. The mixture was brought to the boil and filtered. The medium was distributed in 4 ml. lots and autoclaved at 15 lb. sq. in for 10 min. Before inoculation 0·5 ml. of water and 0·5 ml. of yeast dialysate (Cohen & Wheeler, 1946), diluted 1/8, were added.

Basal medium for testing growth factor requirements (*B* 1). This was the same as the modified C.W. medium without the yeast dialysate. In some experiments the soluble starch was omitted and a Seitz-filtered solution of crystalline bovine albumin (Armour), to give a final concentration of 0·1 % (w/v), was added after the rest of the basal medium had been autoclaved.

Basal medium for testing amino acid requirements (*B*2). This was prepared in the same way as the modified C.W. medium but 3·7 g. NaCl/l. was added in place of the casein hydrolysate, and the following growth factors in place of the yeast dialysate: 10^{-5}M-nicotinic acid, 6 μg. guanine/ml., 6 μg. hypoxanthine/ml., 6 μg. xanthine/ml., 1·0 μg. haemin/ml., 50 mμg. biotin/ml. (final concentrations).

Growth factors and amino acids. These were commercial preparations obtained from British Drug Houses Ltd., Roche Products Ltd., and L. Light and Co. Ltd. Solutions were sterilized by autoclaving at 15 lb. pressure for 10 min.

EXPERIMENTAL

Growth factor requirements

Tests were made with the following 19 growth factors, using the concentrations shown in Table 1: biotin, calcium pantothenate, riboflavin, thiamine hydrochloride, *p*-aminobenzoic acid, haemin, pyridoxine, folic acid, choline chloride, spermine phosphate, guanine, adenine, xanthine, hypoxanthine, cytosine, uracil, thymine, inositol and nicotinic acid. Various mixtures of growth factors were prepared and 0·5 ml. of each mixture was added to 4 ml. of basal medium B1 containing either starch or albumin, and the volume made up to 5 ml. Each tube was inoculated with approximately 10^3 organisms, and the growth recorded each day. Modified C.W. medium was also inoculated and used as a control.

Table 1. *Tests for growth factor requirements of* Haemophilus pertussis

Each tube contained basal medium B1 and growth factors to a total volume of 5 ml. Concentrations of growth factors (μg./ml. medium): 5×10^{-3} biotin, 1·0 calcium pantothenate, 0·25 riboflavin, 0·25 thiamine hydrochloride, 0·1 *p*-aminobenzoic acid, 1·0 haemin, 1·0 pyridoxine, 0·1 folic acid, 2·0 choline chloride, 1·0 spermine phosphate, 5·0 guanine, 5·0 adenine, 5·0 xanthine, 5·0 hypoxanthine, 5·0 cytosine, 5·0 uracil, 5·0 thymine, 5·0 inositol. Nicotinic acid 10^{-5}M. Inoculum: 10^3 cells. Growth as extinction after 4 days.

	Strain 724		Strain 3256	
Growth factor omitted	Starch	Albumin	Starch	Albumin
	Growth			
All	0·01	0·02	0·00	0·02
None	0·76	0·69	0·70	0·73
Purines	0·27	0·42	0·39	0·46
Nicotinic acid	0·02	0·03	0·01	0·00
Any of the other factors	0·85–0·51	0·68–0·51	0·78–0·56	0·78–0·57
Control (yeast extract)	0·76	0·54	0·55	0·54

The results (Table 1) show that a mixture of the 19 growth factors gave as good or better growth than yeast dialysate, that the omission of nicotinic acid completely prevented growth, and that the omission of the purines decreased but did not prevent growth. Omission of other factors produced slight variations in growth, but no one appeared to be significant.

Nicotinic acid. Titration of nicotinic acid and nicotinamide, with the other factors present at the concentrations in Table 1, showed that full growth was obtained with 4×10^{-7} M of either.

Purines. When added to the basal medium B1 with all the other factors given in Table 1, guanine, hypoxanthine or xanthine at 5 μg./ml. all produced equal stimulation of growth. Adenine alone produced neither stimulation nor inhibition, but added together with the other purines it decreased their stimulating effect. To determine the optimum level of purines a mixture was prepared containing equal quantities of guanine, xanthine and hypoxanthine, and dilutions of this were added to the basal medium B1 containing nicotinic acid. A total purine content of 18 μg./ml. gave a growth at 93 hr. which was twice as dense as that without the purines, but higher levels produced only a further slight increase.

Biotin and haemin. The basal medium B1, with starch, was supplemented with the optimal levels of nicotinic acid, guanine, xanthine and hypoxanthine and to this were added mixtures of the other growth factors from which each was omitted in turn. Under these conditions the omission of biotin and haemin consistently reduced growth in the early stages, whereas the omission of the other factors was without effect. This early stimulation by biotin and haemin was most evident with freshly isolated strains (Table 2).

Table 2. *Effect of biotin and haemin on the growth of freshly isolated strains*

Each tube contained basal medium B1 and growth factors to a total volume of 5 ml. Concentration of growth factors. Nicotinic acid 10^{-5} M. Purines: guanine, xanthine and hypoxanthine each at 6 μg./ml. Biotin 50 mμg./ml. Haemin 1·0 μg./ml. Inoculum 10^8 cells. Growth as extinction after 4 days. Experiment performed within 11 days of taking swab from patient.

	Growth					
Strain	Basal medium B1	B1+yeast extract	B1+growth factors	B1+nicotinic acid	B1+nicotinic acid and purines	B1+nicotinic acid, purines, biotin and haemin
6294	0·00	0·32	0·44	0·21	0·37	0·66
3886	0·00	0·53	0·72	0·33	0·58	0·77

Other strains. The findings in Table 1 were confirmed with four other dried strains and five strains tested within 11 days of taking the swab from the patient.

Amino acid requirements

Tests were made with the following 17 amino acids, using the concentrations shown in Table 3: L-alanine, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, DL-serine, DL-threonine, L-tyrosine, and L-valine.

Media were prepared by adding various mixtures of amino acids to the basal medium B2. Tubes of media were inoculated with *c.* 10^3 cells and the growth recorded each day. A medium containing casein hydrolysate in place of the amino acids was used as a control.

The results recorded in Table 3 show that the medium containing all 17 amino acids supported growth but not as well as the control medium; the omission of glycine prevented growth; the omission of glutamic acid or cystine restricted growth markedly, and the effect of omitting leucine or isoleucine was different with different strains.

Table 3. *Tests for amino acid requirements of* Haemophilus pertussis

Each tube contained basal medium B2 and amino acids to a total volume of 5 ml. Concentration of amino acids ($\times 10^{-4}$M): 2·7 L-alanine, 8·0 L-arginine, 6·0 L-aspartic acid, 1·0 L-cystine, 100·0 L-glutamic acid, 3·0 glycine, 1·7 L-histidine, 4·0 L-isoleucine, 6·0 L-leucine, 5·0 L-lysine, 4·0 L-methionine, 0·5 L-phenylalanine, 7·3 L-proline, 2·0 DL-serine, 2·0 DL-threonine, 4·0 L-tyrosine, 1·5 L-valine. Inoculum: 10^3 cells. Growth as extinction after 5 days.

	Strain		
Amino acid omitted	724	446	17790
	Growth		
None	0·68	0·79	0·76
Glutamic acid	0·13	0·11	0·10
Glycine	0·02	0·08	0·05
Leucine	0·59	0·71	0·35
Isoleucine	0·24	0·66	0·54
Cystine	0·17	0·16	0·15
Any of the other acids	0·79–0·61	0·88–0·72	0·84–0·72
Control (stock casein hydrolysate)	0·96	0·97	0·95

A medium was also prepared containing a mixture of amino acids of approximately the same composition as casein hydrolysate (based on the data of Block & Bolling, 1951). This, however, did not support the growth of small inocula, nor did a medium containing the mixtures of amino acids used by Hornibrook (1940) or Pollock (1949).

Freshly isolated strains. Six strains tested within 13 days of taking the swab from the patient grew in media containing amino acid mixtures at the same rate as the dried strains.

Replacement of glutamic acid. It is evident from Table 3 that glutamic acid was not essential for the growth of *Haemophilus pertussis.* Since, however, glutamic acid was the amino acid present in the highest concentration and since it was previously shown (Jebb & Tomlinson, 1951) that it was most rapidly oxidized by resting suspensions, it is probably the principal energy source for growth in these media. It was also shown previously that aspartic acid, proline, alanine, succinate and lactate were oxidized by resting suspensions. To determine whether these substances could replace glutamic acid, a basal medium was prepared containing all the amino acids in Table 3 except glutamic acid and to tubes of this were added glutamic acid, proline, aspartic acid and alanine at 0·01 M final concentration and succinate and lactate at 0·02 M. Glucose, which was not oxidized, was also added to a tube of the basal medium at 0·01 M. Tubes were inoculated with *c.* 10^3 cells. The growth with aspartic acid, alanine, proline and lactate was 30–60 % of that with glutamic acid, whereas growth with glucose and succinate was no greater than that in the basal medium.

Growth in simplified media. To determine whether a single amino acid could act as a sole source of energy, carbon and nitrogen, media were prepared with salts, starch, cystine and growth factors as in previous amino acid experiments, and either glutamic or aspartic acids or proline or alanine at 0·01 M final concentration. Tubes were inoculated with *c.* 10^6 and *c.* 10^3 cells. The medium containing alanine did not support growth, only large inocula grew in the medium containing proline, whereas small inocula grew slowly in media containing glutamic acid or aspartic acid.

Antagonism between amino acids. As the organism grew in a medium containing only cystine, glutamic or aspartic acid, salts and starch, it was apparent that glycine, isoleucine and leucine were not essential for growth. The reduction of growth on omitting these acids from mixtures (Table 3) was presumably due to inhibition by other amino acids. Preliminary investigations showed that two series of amino acids were involved: (1) isoleucine, leucine and valine, and (2) glycine, methionine, proline and serine. Two experiments were performed omitting the appropriate amino acids singly and in combination (Table 4). The results of Expt. 1 showed that valine was inhibitory and that the inhibition was counteracted by isoleucine or leucine, but most strongly by both together. From the results of Expt. 2 it appeared that glycine overcame an inhibition caused by methionine, proline and serine in combination. Slight inhibition was produced in the absence of glycine by these acids singly or in pairs, but the full effect was only observed when all three acids were present together.

Table 4. *Antagonisms between amino acids*

Each tube contained basal medium B2 and amino acids to a total volume of 5 ml. Concentrations of amino acids ($\times 10^{-4}$ M): 2·7 L-alanine, 8·0 L-arginine, 6·0 L-aspartic acid, 1·0 L-cystine, 100·0 L-glutamic acid, 3·0 glycine, 1·7 L-histidine, 4·0 L-isoleucine, 6·0 L-leucine, 5·0 L-lysine, 4·0 L-methionine, 0·5 L-phenylalanine, 7·3 L-proline, 2·0 DL-serine, 2·0 DL-threonine, 4·0 L-tyrosine, 1·5 L-valine. Inoculum: 10^3 cells. Growth as extinction after 5 days.

Experiment 1		Experiment 2	
Acid omitted	Growth	Acid omitted	Growth
None	0·45	None	0·35
Isoleucine	0·31	Glycine	0·03
Leucine	0·30	Methionine, proline and serine	0·25
Valine	0·53	Glycine, methionine, proline and serine	0·21
Isoleucine and leucine	0·13		
Isoleucine, leucine and valine	0·26		

Racemic amino acids. During experiments to devise the medium described in Table 3, it appeared that growth was slower in media containing racemic amino acids than in those containing the L-acids. An experiment in which three strains were inoculated into media prepared with racemic and L-acids showed that growth was consistently better in media prepared with the L-acids.

DISCUSSION

This study differs from most of the previous work on the growth requirements of *Haemophilus pertussis* in that small washed inocula (*c.* 200 cells/ml.) were used throughout as compared with 4×10^6/ml. used by Ungar *et al.* (1950); 3 billion/5 ml. by Watanabe *et al.* (1952); 2×10^7/ml. used by Farrell & Taylor (1945); and 1 loopful of suspension (1 mg./ml.) in 2 ml. used by Imamura (1952).

The only vitamin absolutely required by the 16 strains used in this work was nicotinic acid (or nicotinamide) and none of them had a requirement for thiamine or a complex supplement as found by Farrell & Taylor (1945). Growth was stimulated by guanine, xanthine and hypoxanthine but not by adenine, riboflavin, *p*-aminobenzoic acid or pyridoxine, in contrast to the findings of Watanabe *et al.* (1952). The early growth of freshly isolated strains was stimulated by biotin and haemin, but this effect was only slight with the dried strains.

No one amino acid appeared to be essential for the growth of *Haemophilus pertussis*. In contrast with the findings of Ungar *et al.* (1950) omission of cystine limited but did not prevent growth, so that the organism could apparently use methionine or sulphate as source of sulphur. Imamura (1952) reported good growth with hyposulphite or thioglycollate and slight growth with methionine, taurine or sodium sulphite, but no growth with sulphate. In a casein hydrolysate medium, the glutamate appeared to serve as the source of energy, and in a mixture of amino acids glutamate could be replaced by alanine, proline, aspartic acid or lactate.

As in the case of other organisms (Gladstone, 1939; Washburn & Niven, 1948; Rowley, 1953) the balance of amino acids was important. Valine in the absence of isoleucine and leucine was inhibitory, and so were methionine, proline and serine, in combination, in the absence of glycine. Dawson, Farnworth, McLeod & Nicholson (1951) reported inhibition of *Haemophilus pertussis* by high concentrations of serine.

It is thus evident that *Haemophilus pertussis* does not have elaborate nutritional requirements. The difficulties frequently encountered in growing this organism are probably due to inhibitors in the medium (Pollock, 1947, 1949; Fisher, 1948).

We are indebted to Mr H. A. Tarr for technical assistance.

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The Acquisition of Penicillin Resistance by *Staphylococcus aureus*, Strain Oxford

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SUMMARY: The *Staphylococcus aureus* strain known as Staph. Oxford (NCTC 6571) produces, in any one mutational step towards penicillin resistance, a number of mutants, differing by various characteristics. One of these characteristics is the degree of penicillin resistance. The most resistant first-step mutant is about five times more resistant than the least resistant one. As such mutants are picked up from different penicillin concentrations, according to their higher or lower resistance, the resultant cultures may appear graded by the amount of penicillin present in the medium from which they were obtained. Mutants recognizable as members of one clone, however, show the same resistance from whatever concentration of penicillin they are isolated, within the range on which their growth is possible.

Another characteristic is the distribution of penicillin sensitivity in the population; it differs between mutant strains but, contrary to expressed views, the curves of distribution do not become flatter in mutants of increased resistance, provided one uses a logarithmic scale for the drug concentrations.

Prolonged culture on agar with sub-inhibitory concentrations of penicillin does not lead to increased penicillin resistance. In fluid medium with low penicillin concentrations, on the other hand, stable mutants, resistant to inhibitory concentrations, soon arise.

The growth rate of most penicillin-resistant strains is slower than that of the original strain, and is slowed down still further by the presence of penicillin, even by less than one-hundredth of the inhibitory concentration.

Variants and mutants of slightly increased penicillin-resistance are often pigmented.

Penicillin in sub-inhibitory concentrations enhances autolytic processes in the original organism and in all the mutants, and curious pictures of colony morphology are encountered which result from the interplay of autolysis and secondary growth.

Demerec (1945, 1948) described the acquisition of penicillin resistance by *Staphylococcus aureus*. He demonstrated that the process was step-wise, and he brought evidence for its mutational character. Most workers accept these two fundamental features. Yet one can recognize a mutation only if the limits of phenotypic variation in a clone and the proportion of its population at every distance from the mean is known, in short, if the distribution of drug sensitivity in the clone is known. In the following, I have attempted to study some quantitative aspects of the process of the acquiring of drug resistance by a population of organisms.

METHODS AND MATERIALS

The organism used throughout this work was the strain of *Staphylococcus aureus*, NCTC 6571, which is referred to here as Staph. H. The media used were nutrient broth and nutrient agar. The plate counts were prepared by pipetting

0·1 ml. of an overnight broth culture, or an appropriate dilution of it, on to the surface of an agar plate and spreading over the entire surface with a glass spreader. The endeavour was made, mostly with success, to use an inoculum which would give counts of 100–300 colonies on the plates which served as controls. On penicillin plates with few 'survivors', that is, cells able to form colonies, the probable error, arising from the small counts, is rather great. This could not be avoided in the individual experiment without making it unwieldy. But every type of experiment was put up repeatedly, with only slight variations, and it seems justifiable to draw conclusions from such results pointing in the same direction. The counts were taken after 3 days incubation at 37°. No further colonies became visible on penicillin agar after that period. Where inocula of 10^5 or more were used, a background of microscopic colonies was often present, but only colonies seen with the naked eye were counted. The symbols employed for the organisms of higher resistance each denote one particular mutant, not a class of mutants, the subscript being the level at which the mutant was selected.

The question arises how best to record the degree of penicillin resistance in a culture. With small inocula complete inhibition is obtained at a much lower penicillin concentration than with large inocula. Plotting the number of 'survivors', that is colonies, against the antibiotic concentration gives, with small inocula, a sigmoid curve corresponding to a normal distribution of resistance. Plotting the logarithms of the numbers of survivors against the logarithm of the penicillin concentration gives curves which terminate steeply over a narrow range of concentrations. For practical purposes the antibiotic concentration giving only 10% survival (i.e. 1·0 below the logarithm of the number inoculated) may be used as a measure of the resistance of the organism. It can be determined by plating from a single dilution of the inoculum with a standard deviation very little greater than the square root of the number of colonies counted.

In order to obtain true mutations for further experiments on the range of resistance of small inocula, much larger inoculations were made on plates containing concentrations of penicillin much above that completely inhibiting the growth of small inocula. The mutant strains thus isolated had their range of resistance determined by investigating the survival of small inocula, as was done for the original parent strain.

RESULTS

The steps towards penicillin resistance

Whereas the penicillin concentration for 10% survival of *Staphylococcus aureus*, strain H, was 0·027 unit/ml., the first-step resistant populations gave resistances from 0·03 to 0·15 unit/ml. These first-step resistant mutants differ not only in the degree of their penicillin resistance, but also in their colony morphology. Colonies that are morphologically alike give, as a rule, populations with the same degree of resistance. One may conclude, therefore, that they are members of a clone, or else that there are a limited number of first-step mutations, each associated with a characteristic colony morphology.

Organisms of higher resistance than the first-step were obtained by culturing, in turn, a large inoculum of the first-step mutant $H_{0\cdot15}$ on to penicillin concentrations above its normal range (small inocula), and so on. After resistance to 1 unit/ml. had thus been reached, it seemed impossible to obtain mutants of still higher resistance by the same method. Although large inocula from a culture resistant to 1 unit/ml. gave growth on agar containing 7, 8 or 9 units/ml., colonies from these plates, subcultured into broth and tested for penicillin resistance, showed only the same degree of resistance as the culture from which they had been derived; or, if they had a slightly higher resistance, it was lost after a couple of subcultures on penicillin-free medium. However, when the culture resistant to 1 unit/ml. was inoculated into nutrient broth containing 10 units/ml., growth appeared after 3 days' incubation, and this on subculture proved resistant to 15 units/ml. It was the same phage type as the original and, therefore, unlikely to be a contaminant.

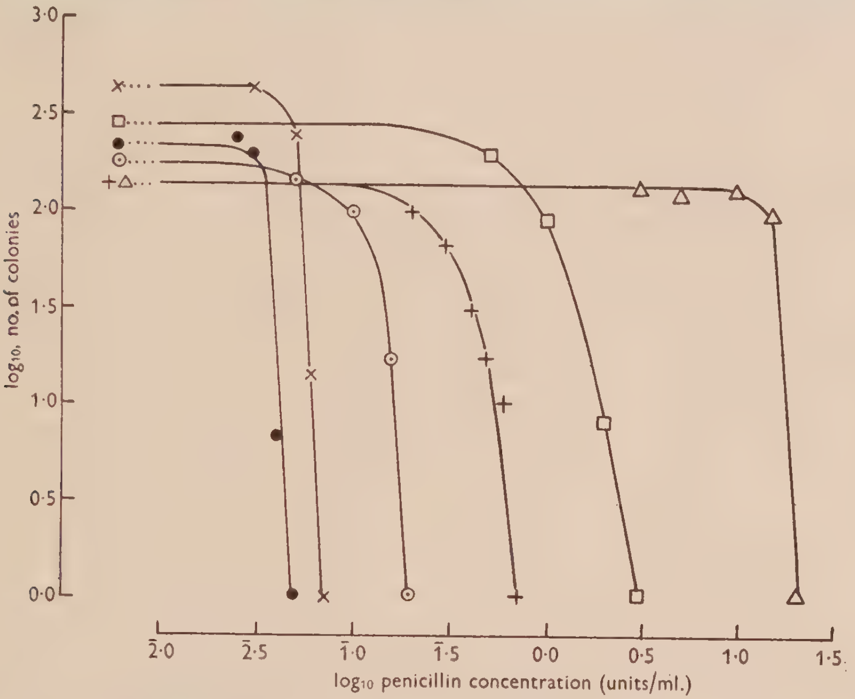


Fig. 1. Curves of decline of viable counts on graded concentrations of penicillin for a broth culture population of Staph. H, two first-step, one second-step, one third-step and one fourth-step mutant. ●, Staph. H, original; ×, Staph. H orange; ⊙, Staph $H_{0\cdot15}$; +, Staph. $H_{0\cdot4}$; □, Staph. $H_{1\cdot2}$; △, Staph. H_{10}.

The consecutive steps of resistant mutants, isolated from the first-step mutant $H_{0\cdot15}$, had their 10% survival at 0·3, 1 and 15 units/ml., respectively (Fig. 1). These are roughly of the same order of magnitude as Demerec recorded for

Staphylococcus aureus NLLR. As the mutants of any one step are not identical, one could probably reach the highest resistance by more steps and also by less, but in the present investigation, as also in Demerec's experiments, only one such series was built up.

The distribution of penicillin resistance in a population

There are considerable differences in the distribution of drug resistance in different strains. Fig. 2 gives the curves for survivors of Staph. H and of five first-step resistant mutants on graded penicillin concentrations. The peculiarities of each strain, with regard to both its level of penicillin sensitivity and the angle at which the curve of survivors descends, remained roughly constant through several subcultures. The break in the curve marked by open circles, however, may not be significant, as the actual counts are rather small numbers at this point.

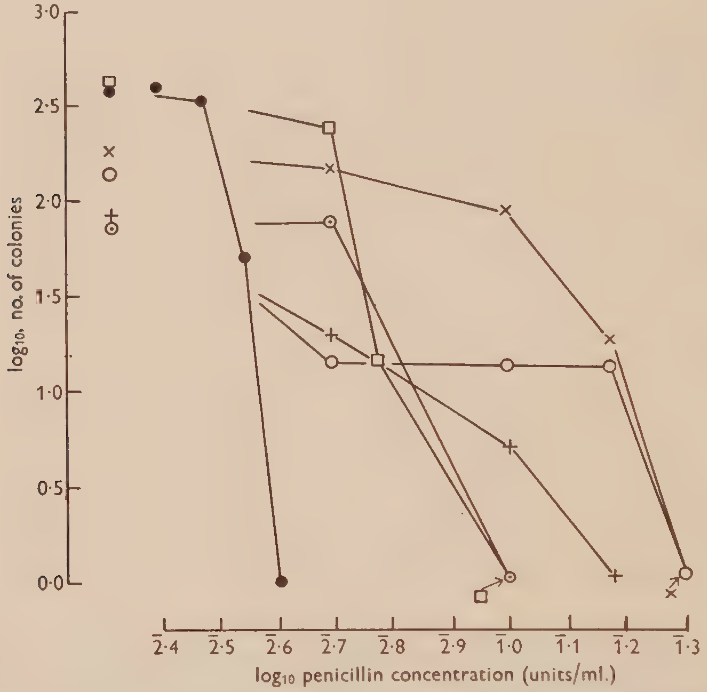


Fig. 2. Curves of colony counts on graded concentrations of penicillin for Staph. H and five first-step resistant mutants. ●, original Staph. H; □, ×, ○, +, ⊙, first-step mutants.

The frequency of mutation

For the original Staph. H, the smallest inoculum of an overnight broth culture which contained resistant mutants was about 40,000. In this relatively small inoculum only one type of mutant was found with sufficient frequency to be picked out regularly. This mutant was deeply pigmented, with a resistance only slightly above that of Staph. H. Non-pigmented mutants were less frequent, being hardly ever found in inocula below 10^5.

The 'grading' of penicillin resistance in relation to the concentration of antibiotic on which the growth has taken place

Eagle, Fleischmann & Levy (1952) found that colonies obtained from plates which contained graded concentrations of an antibiotic presented a continuous spectrum of stable resistances, with no evidence of a step-like process. If large inocula of 10^6 or more were used—Eagle *et al.* used 5×10^7—this grading could indeed be observed (Table 1). If, on the other hand, one used an inoculum of a size unlikely to contain mutants, organisms from penicillin plates were no more resistant than the original culture (Table 2). From large inocula on low penicillin concentrations one could isolate both normal 'overlaps', that is colonies giving cultures no more resistant than the parent strain, and mutants,

Table 1. *The penicillin resistance of Staph. H and of three colonies obtained by plating an inoculum of* 10^6 *of Staph. H on* 0·027, 0·035 *and* 0·05 *unit/ml. respectively*

Numbers represent number of colonies growing from equal inocula.

Penicillin content of agar (unit/ml.)	Strain			
	H	0·027	0·035	0·05
None	94	94	76	92
0·025	100	49	93	85
0·03	1	3	71	93
0·035	1	3	8	92
0·04	—	—	—	97
0·05	—	—	—	48
0·06	—	—	—	14

Table 2. *The penicillin resistance of Staph. H and of four colonies, two of which had grown on* 0·025 *unit/ml. and two on* 0·027 *unit./ml.*

The colonies of each pair differed in that one was from a plate with an inoculum of 10^2, the other from a plate with an inoculum of 10^6. Numbers represent number of colonies growing from equal inocula.

Penicillin content of agar (unit/ml.)	Strains				
		Large inoculum		Small inoculum	
	H	$0{\cdot}025_1$	$0{\cdot}027_1$	$0{\cdot}025_3$	$0{\cdot}027_3$
None	109	72	101	104	90
None	121	77	C	99	73
0·025	121	100	C	78	47
0·027	13	87	118	26	1
0·03	1	77	108	2	1
0·035	—	2	106	—	—
0·04	—	—	9	—	—
0·05	—	—	—	—	—

C = contaminated

as a comparison of the two colonies from 0·027 unit/ml., used in the experiments of Tables 1 and 2 respectively, shows. But even penicillin concentrations that are barely inhibitory if tested with small inocula, exercise a considerable selective influence in large ones. The colonies $0{\cdot}027_1$ and $0{\cdot}025_1$ were, with a few dozen others, marked on the plates after 1 day incubation. Being resistant mutants and, therefore, able to start growth relatively early, they appropriated the field, thereby reducing the chances for the normal overlaps, of which just a few hundreds had grown up by the third day. The culture $0{\cdot}027_1$ employed in the experiment of Table 1 had been derived from such a late colony, which was an overlap of the normal population of Staph. H.

It was thought possible that, in any one mutant, the actual degree of resistance might be influenced by the concentration of penicillin with which the organisms had been in contact. To test this, organisms that were members of the same mutant clone, but grown on different penicillin concentrations, had to be examined. Unfortunately, in most instances the colony morphology of organisms on different penicillin concentrations may not allow the recognition of members of the same clone. The exception is the pigmented mutant which turns up with great regularity and stands out well. Staph. H was inoculated—inoculum 10^6—on to a series of penicillin plates; on 0·025 unit/ml. seven yellow colonies grew up amongst various others, on 0·027 unit/ml. five and on 0·03 unit/ml. two. One yellow colony from each of these concentrations was picked into broth and the resulting cultures tested for penicillin sensitivity. They were all a small but definite step more resistant than the original Staph. H. Although the three yellow colonies had grown on different concentrations of penicillin, the broth cultures derived from them were exactly alike in their penicillin sensitivity (Table 3).

Table 3. *The penicillin sensitivity of yellow colonies grown on* 0·025 *unit/ml.,* 0·027 *unit/ml. and* 0·03 *unit/ml. respectively*

Numbers represent number of colonies growing from equal inocula.

Penicillin content of agar (unit/ml.)	Strains			
	H	$Y_{0\cdot025}$	$Y_{0\cdot027}$	$Y_{0\cdot03}$
None	62	50	72	59
None	53	64	69	67
0·025	57	58	77	61
0·03	—	49	67	64
0·035	—	56	60	48
0·04	—	23	39	17
0·05	—	—	—	—
0·06	—	—	—	—

The drug resistance in populations grown in low drug concentrations

Small inocula, unlikely to contain mutants, were plated on sub-inhibitory or slightly inhibitory concentrations of penicillin. Colonies from these plates were picked into broth, and the resulting populations examined for distribution of penicillin resistance. Colonies that had grown for 1, 2, 3 and 4 days respectively on a plate containing 0·027 unit/ml. gave precisely the same curves for survivors as the original culture (Table 4). As all the colonies had been picked into broth without penicillin, and the survivor curve determined on the broth culture population, it was thought possible that during growth in the penicillin-free medium a culture with a higher resistance might revert to the original level. Therefore, another series of experiments was done, in which the colonies themselves were cut out with the underlying agar, suspended in broth, and tested directly for penicillin resistance. Some colonies were tested after subculturing on penicillin agar from single colonies daily for 4 days. The penicillin concentrations of the plates were 0·02, 0·025 and 0·03 unit/ml. respectively. No displacement of the mode towards higher drug concentrations was ever encountered.

Table 4. *The penicillin resistance of colonies grown for varying times on nutrient agar containing* 0·027 *unit penicillin/ml.*

Numbers represent number of colonies growing from equal inocula.

	Source of inoculum				
		Staph. H, grown on 0·027 unit/ml. for:			
Penicillin content of agar (unit/ml.)	Staph. H, broth culture	1 day	2 days	3 days	4 days
None	346	276	370	435	200
None	339	283	312	430	182
0·027	346	306	287	394	190
0·03	318	305	341	393	177
0·032	70	55	152	62	37
0·035	—	—	—	—	—

Table 5. *The penicillin resistance of populations of Staph. H grown in nutrient broth, and in broth containing* 0·02 *unit penicillin/ml.*

A, culture in nutrient broth, 4 days old; B, culture in nutrient broth, 1 day old, after four daily subcultures in nutrient broth; C, culture in nutrient broth with 0·02 unit/ml., 4 days old; D, culture in nutrient broth with 0·02 unit/ml., 1 day old after four daily subcultures in the same medium. Numbers represent number of colonies growing from equal inocula.

Penicillin content of agar (unit/ml.)	A	B	C	D
None	96	67	117	118
0·02	73	25	105	116
0·03	1	—	36	118
0·04	—	—	13	35
0·05	—	—	1	1

The results were entirely different if small inocula were grown in sub-inhibitory concentrations of penicillin incorporated into broth instead of agar. With or without repeated subculture, these populations showed a marked increase in resistance (Table 5).

Pigmentation and drug resistance

While Staph. H is almost completely white, deeply pigmented mutants can easily be obtained from an inoculum of the order of 10^4 and, as they possess a slight but clearcut higher resistance to penicillin, they can be selected on media containing penicillin just slightly inhibitory for the original culture. They were obtained on numerous occasions by growing a small inoculum of Staph. H, from a one-colony isolation, in broth with 0·025 unit/ml.; but also by plating an inoculum of appropriate size on penicillin agar.

Not with the same regularity, but still on several occasions, colonies of a lesser degree of pigmentation and a resistance to penicillin intermediate between that of Staph. H and the deeply pigmented mutant were observed (Table 6).

Table 6. *The penicillin resistance of Staph. H and of two of its pigmented mutants, selected on a nutrient agar plate on to which a* 30 *hr. culture of Staph. H in broth containing* 0·025 *unit/ml. had been subcultured*

Numbers represent number of colonies growing from equal inocula.

Penicillin content of agar (unit/ml.)	Strains		
	Staph. H	Deep yellow	Light yellow
None	94	272	112
None	96	294	100
0·03	87	315	78
0·04	—	214	23
0·05	—	13	—
0·06	—	—	—

The deeply pigmented variants were not due to the same mutational process on every occasion. The normal, most frequently obtained, mutant was rather stable with regard to both pigmentation and penicillin resistance, and threw off white, penicillin-sensitive, back mutants. But on one occasion a pigmented mutant was observed which reverted to penicillin sensitivity within a few subcultures on penicillin-free medium, without losing its pigmentation; and on another occasion a yellow colony, picked into broth and subcultured from the broth culture on to penicillin-free agar, gave rise to colonies showing a range of yellows with a parallel range of penicillin resistances.

The growth rate of drug-resistant organisms

When both the original culture and some more resistant variant were plated on nutrient agar, it often took a longer time for the resistant strain to form visible colonies. The growth rate of several of these mutants in nutrient broth was, therefore, determined. About 10^4 organisms of an overnight broth culture were inoculated into 10 ml. broth in tubes of 1 in. diameter, and incubated at 37°. 0·1 ml. of these cultures, or of appropriate dilutions, was plated at intervals.

With a few exceptions like $H_{0\cdot15}$, which had the same growth rate as the original Staph. H, the resistant mutants were found to have a longer lag phase and sometimes also a longer generation time than the original strain. Moreover, their maximal viable population usually remained somewhat below that of Staph. H. Fig. 3 gives the growth rates of Staph. H and several mutants in penicillin-free broth. It also shows that the growth rate of a mutant is, in general, lower, the higher its penicillin resistance.

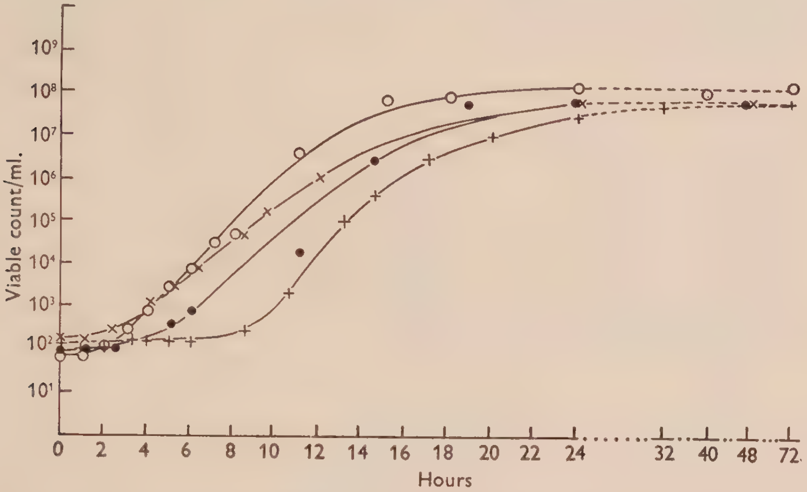


Fig. 3. Growth curves in penicillin-free nutrient broth of Staph. H and of H 'deep yellow', $H_{0\cdot4}$ and H_{10}, incubated at 37°. ○, Staph. H original; ×, Staph. H 'deep yellow'; ●, Staph. $H_{0\cdot4}$, +, Staph. H_{10}.

The growth rate in the presence of penicillin

It was to be expected that penicillin in slightly inhibitory concentrations might influence the growth rate of sensitive organisms, but it was rather surprising to find that the growth rate of mutants of every degree of resistance was markedly lowered in the presence of sub-lethal concentrations of the drug. In these experiments also the inoculum was from an overnight broth culture. About 10^4 organisms were inoculated into 10 ml. nutrient broth to which penicillin had been added, and incubated at 37°. 0·1 ml. of these cultures or appropriate dilutions were plated at intervals.

For H_{10}, for instance, the number of viable organisms was diminished at 15 units/ml., but the growth rate was affected by 0·1 unit/ml. (Fig. 4). This reduced growth rate was mainly due to a prolongation of the lag phase, 0·1 unit prolonged it by about 1 hr. to 7 hr., with 1 unit/ml. it was 9 hr., and with 10 units/ml. 11 hr. In fact, one might be inclined to classify penicillin as a bacteriostatic agent.

A similar experiment, in which about 100 organisms of the mutant H_{10} were inoculated on to agar containing sub-lethal concentrations of penicillin, showed that, while there was no decrease in numbers of colony-forming survivors on agar containing 10 units/ml., the size of the colonies produced was markedly diminished even on 1 unit/ml. agar. The photographs of Pl. 1, fig. 1, were taken after 3 days incubation, but the colonies shown in Pl. 1, fig. 1*b*, *c*, never grew up to normal size, even after several more days incubation.

It was further found that mutants of the same step, with approximately the same degree of resistance to penicillin, and the same growth rate on penicillin-free media, might have entirely different growth rates in the presence of penicillin. The growth rate in the presence of penicillin was as much a differentiating characteristic between mutant strains as the resistance itself, and perhaps even a finer one. Differences in growth rate and/or lag were readily detected by the size of the colonies on agar (Table 7), but they showed clearly also in broth.

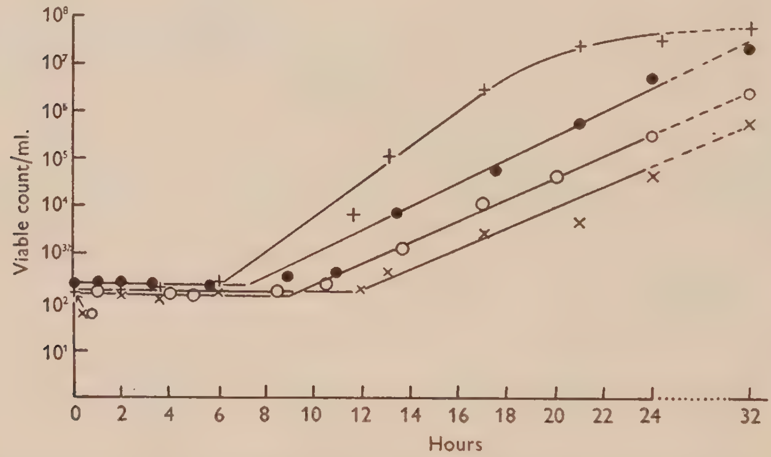


Fig. 4. Growth curves of the penicillin-resistant mutant H_{10} in nutrient broth and in broth with 0·1, 1 and 10 units/ml. penicillin. The inoculum was 10^4 organisms of an overnight broth culture per 10 ml. medium. Incubation at 37°. +, without penicillin; ●, with 0·1 unit/ml.; ○, with 1 unit/ml.; ×, with 10 units/ml.

Autolytic phenomena

Staph. H and all its variants, if subcultured on sub-inhibitory concentrations of penicillin, produce colonies that are rather transparent compared with the opaque colonies on penicillin-free agar. If such plates are left at room temperature for a couple of weeks, some colonies go completely transparent and remain so; these give no growth on subculture. Others go transparent, but then develop luxuriant opaque secondary growth; this hardly ever occupies the whole area of the lysed colony, but shows a fantastic variety of shape, size, opacity and pigmentation (Pl. 1, fig. 2). Frequently, the centre of the colony remains lysed, while a thick opaque wall grows around the periphery. Sometimes the wall is not solid, but formed of separate papillae; or a group of secondary colonies develops in the centre of the lysed one (Pl. 1, fig. 3*a*,*b*,*c*). The secondary growth can be subcultured and is composed of organisms having the same degree of resistance as the inoculum.

Table 7. *The penicillin resistance and size of colonies on penicillin agar of two second-step mutants, both isolated from a plate containing* 0·4 *unit penicillin/ml.*

The inocula were overnight broth cultures. Numbers represent colonies growing from equal inocula; in brackets is the average size of the colonies in mm., measured after 3 days incubation at 37°.

Penicillin content of agar (unit/ml.)	Mutant	
	$H_{0\cdot4}A$	$H_{0\cdot4}B$
None	108 (3·5)	139 (3·5)
None	138 (3·5)	146 (3·5)
0·2	98 (2)	89 (1·2)
0·3	67 (1·5)	66 (1)
0·4	70 (1)	30 (0·6)
0·5	63 (0·8)	17 (0·5)
0·6	36 (0·5)	10 (<0·5)
0·7	10 (<0·5)	—

DISCUSSION

Whether the acquisition of drug resistance is due predominantly to a spontaneous mutational or to a specifically induced adaptive process has been under discussion for many years, and probably will be for many more. On the whole, the observations reported here come down on the mutational side. They show that no stable increase in penicillin resistance arises from inocula too small to contain mutants, and that organisms belonging to one clone have the same penicillin resistance whatever concentration of penicillin they were grown on in the first instance. Grading of resistances in relation to the drug concentration in the medium on which the culture had been grown was observed by Eagle *et al.* (1952), and interpreted as due to an adaptational mechanism. The observation itself has been confirmed in the present investigation, but has to be interpreted as due to the multiplicity of kinds of mutants. Now and then, however, events occur which present a curious mixture of step-wise and gradual changes, as, for instance, the step-wise mutational acquisition of both pigmentation and penicillin resistance, and the continuous graded return to sensitivity and lack of pigmentation, observed on one occasion.

Demerec (1948) noted that, whereas the first steps towards penicillin resistance were always small, the degree of streptomycin resistance acquired in the first step might differ considerably. Welsch (1952) established that, for staphylococci in the process of becoming streptomycin resistant, the differences between the resistance levels of different first-step mutants were 50- to 100-fold. In the investigation here presented the first-step mutants to penicillin resistance differ in a similar way, though to a much lesser extent.

All these mutants have clear-cut individualities of their own, relating not only to the penicillin concentration at which the greatest number of their population is killed, but also to the frequency with which they arise, to their growth rate with and without penicillin, to the phenotypic distribution of penicillin resistance in their populations and, sometimes, to their colony morphology.

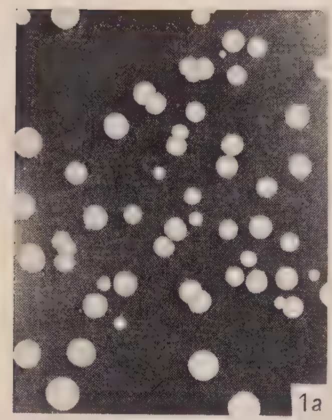
Of these, the phenotypic distribution of penicillin resistance has, so far, received hardly any attention. Collard (1953) noted that, as resistance developed, the modal value of sensitivity moved towards higher drug concentrations, and the scatter about the mode increased. This increased scatter, however, appears only in graphs where a semilogarithmic scale is used. If drug concentrations as well as survivor numbers, are recorded on a logarithmic scale, the most highly resistant organisms may have a smaller or a larger scatter than the original strain.

Yudkin (1953) formulated a theory of 'clonal variation'. According to this theory an organism phenotypically more resistant than the organisms occupying the modal point of the distribution curve will produce a clone with the mode situated at the penicillin concentration to which this organism is resistant. If this were true, accidental and phenotypical characteristics could be inherited, which is contradictory to common experience in other biological fields.

At least for penicillin sensitivity, the staphylococci follow the recognized statistical pattern, and the members of the population, phenotypically more resistant than the mode, produce clones with the mode in the same place as the original strain. Only mutants show the displacement of the mode and Yudkin's experiments can be explained on the assumption that what he really dealt with were mutants.

Gibson & Gibson (1951), working with *Escherichia coli* and streptomycin, obtained a 10-fold increase in resistance by subculturing repeatedly in a synthetic fluid medium containing 2·5 μg. streptomycin/ml. They interpreted this result as adaptation. Also in my experiments the ready increase in resistance could be observed on subculture in fluid medium containing the drug, but not if the drug was incorporated into agar. As there seemed no reason why the organisms should be capable of adaptation in fluid but not on solid medium, another explanation was looked for. Welsch (1952) introduced the concept of an equilibrium in a large population between the resistant mutants and the organisms of the original stock. Sub-inhibitory drug concentrations disturb this equilibrium in favour of the mutants. It is clear that such a mechanism would have a far better scope to operate in fluid medium with its 'redistributed' population than on solid.

The pigmentation of slightly resistant mutants is not confined to penicillin resistance, but has been observed also in connexion with a low-grade sulphonamide resistance. It has often been emphasized that penicillin resistance and sulphonamide resistance occur independently. One of the pigmented mutants obtained on penicillin agar was, therefore, compared with the Staph. H in respect of sulphonamide resistance. Far from showing an increased sulphonamide resistance, it was appreciably more sensitive than the original non-



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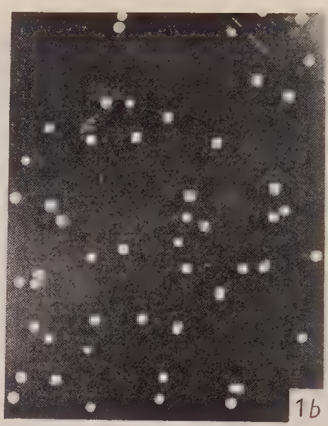

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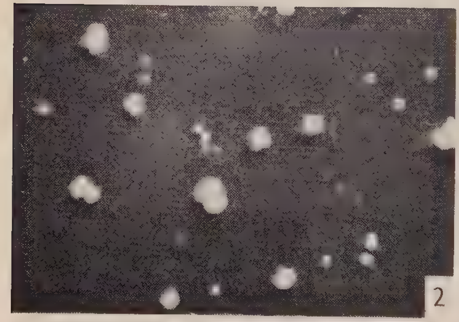

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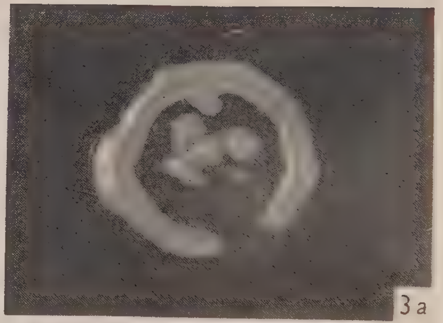

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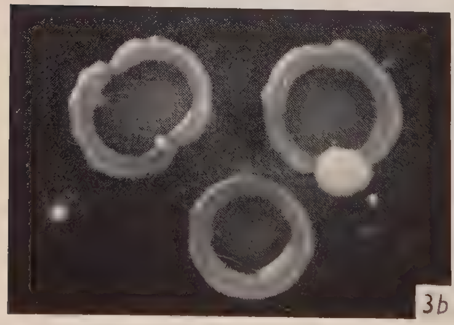

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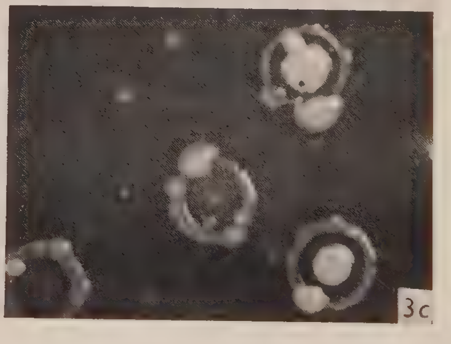

3c

A. Mayr-Harting—Penicillin resistance. Plate 1

(*Facing p.* 21)

pigmented strain (Table 8). One must, therefore, assume, that the pigmentation is a by-product of the cell metabolism, not specifically connected with the by-passing of the penicillin-blocked pathways.

Table 8. *The sulphonamide resistance of Staph. H and of the pigmented mutant* $Y_{0\cdot03}$, *selected on a plate containing* 0·03 *unit/ml. penicillin*

Numbers represent number of colonies growing from equal inocula.

Sulphonamide content of agar	Staph. H	$Y_{0\cdot03}$
Control	96	240
Control	85	272
1:50,000	119	243
1:20,000	93	150
1:15,000	112	101
1:10,000	107	82
1:7,500	122	—
1:5,000	4	—

My thanks are due to Professor K. E. Cooper for his interest in this work and his valuable criticism; to Dr A. H. Linton for drawing the graphs; and to Mr D. White and Mr H. J. Washer for the photographs.

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EXPLANATION OF PLATE

Fig. 1*a, b, c*. Staph. H_{10} on penicillin-free nutrient agar and on agar containing 1 unit/ml. and 10 units/ml. penicillin, respectively. All three plates were inoculated with the same sized inoculum of the same overnight broth culture and incubated at 37° for 3 days. Magnification ×1·2.

Fig. 2. As Fig. 1*c*, photographed 3 weeks later. Some colonies have become very transparent, others have developed secondary opaque growth of irregular nibbled appearance. Magnification about ×2·7.

Fig. 3*a, b, c*. Autolytic colonies with secondary growth of a first-step resistant mutant, having its steepest decline of survivors at 0·06 unit/ml., grown on agar containing 0·05 unit/ml. penicillin. Magnifications, ×8·4, ×5·4 and ×3·6, respectively.

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Cooper, P. D. (1955). *J. gen. Microbiol.* **13**, 22–38

The Site of Action of Penicillin: some Changes in *Staphylococcus aureus* during the First Two Hours Growth in Penicillin Media

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SUMMARY: To see whether the initial damage caused by penicillin involved the osmotic barrier, certain characters of *Staphylococcus aureus* were examined at intervals during the first 2 hr. after addition of penicillin to growing cultures. In the 30 min. following addition of penicillin the cell appeared to expose nearly all its reserve penicillin-binding component (PBC), the penicillin uptake being double that which occurred without growth and the reserve PBC disappearing. The amount of PBC in the cell and its rate of exposure to penicillin in excess of normal synthesis (rate of turnover?) were both very small. Between 30 and 60 min. the uptake of Na, Mg, K, P and increase of total dry matter ceased abruptly. Continued increase in dry wt., while P, Na, Mg and K decreased slightly, resulted in the penicillin-treated cells becoming relatively deficient in these elements. Synthesis of lipid phosphorus and some, but not all, large molecule phosphates still continued, and Fe and Co uptake were not affected. After 60–80 min. water entered and solutes began to leave the cell, and synthesis of large molecule P ceased. The primary site of action of penicillin is probably not concerned with gross assimilation of Na, K, Mg, P, Fe, Co, or any substance contributing more than 10% of the dry wt., or with gross synthesis of lipid P. It may, however, involve a reaction turning over 3000 times while the cell mass is doubled.

To describe fully the mode of action of a drug one must account for all the changes which take place from the moment of first contact between cell and drug. Although this is clearly impossible to do in detail, some such attempt is necessary as it is very difficult to pin-point any particular defect as being the cause of death. The specific nature of the penicillin molecule and the small amounts bound by cells suggest that the changes caused by the drug are sequences of events branching from perhaps only one small and specific injury. Each event plays its part in a sequence which culminates in the inability of most of the population to divide, and the relative time of appearance of abnormalities is important.

It was shown elsewhere (Cooper, 1954) that penicillin is firmly bound in a specific manner by a lipid-containing fraction near the cell wall of a sensitive staphylococcus. Believing that the penicillin-binding component (PBC) of sensitive cells (Rowley, Cooper, Roberts & Lester Smith, 1950) is likely to be the initial site of the bactericidal action of penicillin when acting at its minimal concentration, it was suggested that its presence in this fraction might indicate that the first effect of penicillin is to disorganize some function specific to the cell wall or to the osmotic barrier. To obtain further information on this possibility, penicillin was added to growing cultures of sensitive staphylococci, and the following characters were examined very soon after addition of penicillin: (*a*) the 'phosphate-impermeable volume' (the space enclosed by the osmotic barrier to phosphate) and the volume of the centrifuged cell pad; (*b*) the dry wt. of the 'lipid particle' fraction, and the phosphate of lipid and other fractions; (*c*) ability of the cell to take up and retain the elements it requires from the medium. (These abilities may or may not be related to osmotic barrier function for elements other than P.)

The observations described are therefore attempts to obtain some idea of the sequence of defects occurring during the first hour or two after addition of penicillin, paying particular attention to possible cell wall or osmotic barrier activities which might be the site of action of penicillin.

METHODS

The organism was the penicillin-sensitive (0·02 unit/ml.) *Staphylococcus aureus* used previously (Cooper, 1954), which was capable in March 1954 of reproducibly binding penicillin in the resting state to 0·90 ± 0·1 unit/g. dry wt. It is interesting that the penicillin-binding capacity of this strain has steadily dropped from 2·4 units/g. in 1949 without concurrent change in penicillin sensitivity or colonial appearance, but the average cell size has increased, so that the number of penicillin molecules bound/cell has not changed. The number of cells/g. dry wt. calculated from viable counts was 8×10^{11}, but calculations from the average cell diameter ($1·3\,\mu$) and specific volume (*c.* 5 ml./g.) give about $3·5 \times 10^{12}$ cells/g. dry wt. This figure, itself liable to considerable error, has been used to calculate that about 300 molecules of penicillin are bound per cell in the resting state. The difference from alternative figures for other strains (Maass & Johnson, 1949*a*; Eagle, 1954) is probably largely accounted for by the difficulty in assessing the number of cells/g. dry wt., as uptakes in units penicillin/g. dry wt. were often similar.

Growth conditions. Most of the experiments involved taking serial samples after adding penicillin to a culture growing under standard conditions, with simultaneous samples from a control culture. Tryptic digest agar was inoculated with young cultures, the cells were harvested aseptically after 16 hr. at 36°, washed twice with sterile distilled water and the dry matter of the suspension was determined turbidimetrically by reference to a calibration curve. Samples of such suspensions were added to 100 ml. warm broth contained in 1 l. long-necked B34 round-bottomed flasks plugged with cotton-wool, so that the final concentrations were 0·2, 1·0 or 2·5 mg./ml. These were shaken with *c.* 10 mg. of Silicone antifoam A (Hopkins and Williams Ltd.) at about 5 cyc./sec. on a Microid shaker (Griffin and Tatlock Ltd.) at 36°. These conditions gave a mean generation time of about 1 hr., which with this organism allowed a good separation in time of many of the metabolic defects caused by penicillin.

Penicillin was generally added 1 hr. after inoculation, when the cells were just entering the logarithmic phase, and 10 ml. samples, taken at intervals, were rapidly cooled in ice and washed once in distilled water, so that the final sample contained less than 1/2000 ml. medium. Penicillin was usually used in the lowest concentration at which penicillin binding is complete within a minute or two at 37°, i.e. 0·1 unit/ml. This concentration is also the lowest at which the rate of bactericidal action is maximal (Eagle, 1951), and at which the production of fresh viable units is immediately stopped (Parker & Marsh, 1946; and present work). High concentrations may give rise to other less specific effects of penicillin.

Measurement of PBC. Assay of PBC by use of radiopenicillin and the preparation of radiopenicillin were as described by Rowley *et al.* (1950); radiopenicillin purity was checked by the methods of Cooper, Clowes & Rowley (1954).

Cell volume. Cell-pad volume was determined by centrifuging 0·25 ml. of cell suspension containing about 0·05 ml. of cells for 30 min. at 3500 *g* in tubes of 2·5 mm. internal diameter. A mark was made at the top of the cell mass, and the wt. of mercury occupying the volume in the cleaned and dried tubes determined. The phosphate impermeable volume was determined as that volume not penetrated by $^{32}PO_4$, in presence of excess phosphate and carried out rapidly at 0° so that negligible exchange of ^{32}P could occur between cells and medium. For this, 0·25 ml. of the same cell suspension was added in the cold to 0·1 ml. of 2M-phosphate at pH 7 containing 0·4 μc. ^{32}P/ml. After mixing, the suspensions were at once centrifuged for 5 min. at 5000 *g* in a refrigerated angle centrifuge using tubes 0·6 mm. internal diameter, and two samples of 0·1 ml. of the supernatant were rapidly removed for ^{32}P assay. Samples for 'cell-pad' and 'phosphate impermeable' volumes were thus centrifuged separately at different rates and ionic strengths, and are not comparable with each other. The average cell-pad volume (6–8 ml./g.) is high compared with certain other staphylococci (3·47 ml./g., Gale, 1947; 3·61 ml./g., Mitchell, 1953), but the organism used here was rather larger than average. The data in Fig. 1 suggests that the specific volumes of cells may not be absolute, but probably depend on growth conditions and speeds and ionic strengths obtaining during centrifuging.

Dry matter. Samples of 10–20 mg. cells in weighed 20 ml. tubes were dried at 110° for 16 hr., cooled over P_2O_5 *in vacuo*, stoppered and re-weighed.

Phosphorus determinations. Inorganic phosphate was determined by the method of Fiske & SubbaRow (1925), organic phosphates being first combusted with 'Analytical Reagent' perchloric acid. Cell suspensions were treated with an equal volume of 6% phenol solution for 20 min. at room temperature to obtain the phenol-soluble phosphates, comprising inorganic (PhI) and organic (PhO) fractions which corresponded quantitatively in normal cells of this staphylococcus with the trichloroacetic acid-soluble (AI and AO) and butanol-soluble phosphates prepared by Mitchell's method (1953). The PhI in the supernatant was determined directly without combustion and the PhO was determined as (total phenol-soluble P–PhI). In the whole cells or fractions ^{32}P was assayed by counting samples dried on planchettes, making due allowance for radioactive decay. To determine ^{32}P in the PhO, PhI was first precipitated as NH_4MgPO_4 by addition to 5 ml. samples of 0·1 ml. 20N-ammonia and 0·1 ml. of 5 % (w/v) $MgCl_2 . 5H_2O$, leaving PhO in solution after 16 hr. at 4°. Contaminating PhO in the washed precipitate was less than 5 % PhI. PhI ^{32}P was determined as total phenol-soluble ^{32}P minus PhO ^{32}P. The relative specific activities ('R value', or ratio of observed value of $^{32}P/P$ to that value expected at complete equilibration) were expressed as described by Mitchell & Moyle (1953). The total P and ^{32}P were determined in the residue after phenol treatment, which was taken as representing the large molecule phosphate.

Determination of other elements. Iron was determined as ^{59}Fe and by the thiocyanate colorimetric method. The samples were combusted to 0·1 ml. with $HClO_4$ and 0·1 ml. 10 % (v/v) HNO_3 and 0·5 ml. water were added; the samples were cooled, mixed with 3 ml. acetone and 0·5 ml. of 20 % (w/v) NH_4CNS, and made up to 5 ml. and the Fe assayed after 30 min. on a Hilger absorptiometer using a blue filter. Sodium, potassium, magnesium and calcium were determined with the Hilger 'Uvispek' photoelectric spectrophotometer using a flame photometer attachment and the quartz prism. Standards contained approximately the same proportions of Na, K, Ca, Mg and PO_4 as found in the samples, and the experimental conditions shown in Table 1 were used (compare Kapuscinski, Moss, Zak & Boyle (1952) for Mg and Ca). Co was determined as ^{60}Co, and by the arc spectrograph.

Viable counts were made by the method of Miles & Misra (1938).

Table 1. *Experimental conditions used for flame photometer assays*

Calibration curves for K and Mg were not linear.

Element	Wave length (A)	Minimum standard (μmole/ml.)	Min. conc. determined (μmole/ml.)	Slit width (mm.)	Pressure H_2 (lb./sq.in.)
Na	5890	0·2	0·02	0·4	1
K	7700	0·5	0·05	0·6	1
Mg	3820	2	0·2	0·8	2
Ca	5540	1	0·1	0·6	1

RESULTS

Changes in cell volume during growth in presence of penicillin

The well-known swelling of organisms in the presence of lethal amounts of penicillin is unaccompanied by increase in cell mass (Fig. 1), and therefore differs from the formation of 'giant forms' in sub-inhibitory titres (e.g. Fleming, Voureka, Kramer & Hughes, 1950) where increase in cell mass occurs but division is inhibited. Fig. 1 shows a progressive decrease in cell-pad and phosphate-impermeable volumes of normal cells during growth under the conditions used here, and penicillin had very little effect on these volumes up to about 60 min. after its addition. However, soon after the dry matter ceased to increase there was a marked increase in the phosphate-impermeable volume of the cells, and a somewhat smaller increase in the cell-pad volume. The changes in cell-pad volume may reflect changes in cell-packing (perhaps due to changes in mutual adhesiveness) rather than in the volume of individual cells, but the similar nature of the changes in phosphate-impermeable volume suggests that this is not so. The important point is that growth in the presence of penicillin causes no rapid collapse in the cell-pad and phosphate-impermeable volumes similar to that produced by addition of butanol or trichloroacetic acid (Mitchell, 1953). About 60 min. after the addition of penicillin, the apparent increase of these volumes without corresponding increase in dry matter suggests that the cells rapidly took up a considerable amount of water. This, with the slow liberation of PO$_4$ and decrease of dry matter which commences soon after, suggests that an increase in the permeability of the osmotic barrier occurs at this stage. Alternatively, the cell wall may become more elastic due to loss of PGP, the polyglycerophosphate complex described by Mitchell & Moyle (1951*b*), or some internal breakdown of large molecules may cause a rise in the internal osmotic pressure. Therefore, at most, breakdown of the osmotic barrier's passive properties has only just begun by 60–100 min. after addition of penicillin.

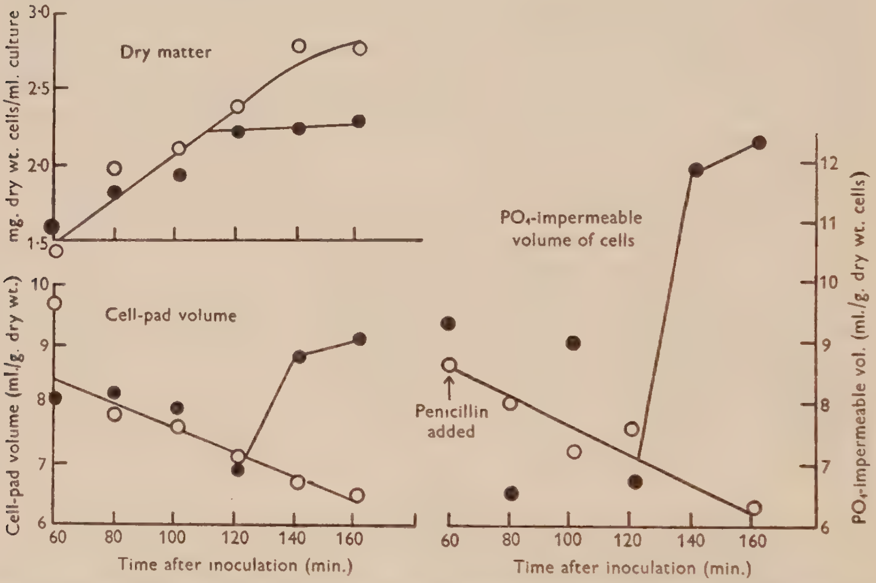

Fig. 1. Changes in cell-pad and phosphate-impermeable volumes of *Staphylococcus aureus* on growth in the presence of penicillin. The washed cells (equivalent to 1 mg. dry wt./ml.) were added to warm shaken broth and penicillin (to 0·1 unit/ml.) was added 60 min. later. Samples were taken at intervals. The phosphate-impermeable and cell-pad volumes are not comparable with each other, as different methods of centrifuging were employed. Normal culture, ○—○; penicillin-treated culture, ●—●.

Changes in cell dry weight

In agreement with the observations of Parker & Marsh (1946), 0·1 unit penicillin/ml. was found to be the lowest concentration at which production of fresh viable units ceased immediately on addition of penicillin. In spite of this, seven separate experiments, including those of Figs. 1 and 4, showed that dry matter increased in identical manner to that of the control for 54–60 min.

after addition of penicillin, when the increase suddenly ceased. (The fourth 'penicillin' point was low in the dry wt. curve of the experiment shown by Fig. 2.) This was similar to the experience of Park (1951), where increase in turbidity, dry weight, nitrogen, phosphorus or nucleic acid was almost normal for 45 min. after addition of penicillin. This time was very much the same for many experiments, with the exception of one (Table 2), although the control cultures took from 57 to 300 min. to double their weight from the time of adding penicillin. The abruptness of the cessation of the uptake of materials comprising the bulk of the dry matter of the cell, namely carbon and nitrogen compounds, indicates that they all cease to enter the cell at about the same time. The dry matter remained unchanged for a further 60 min. and then gradually decreased.

Table 2. *The lack of effect of penicillin on iron uptake*

Washed freshly harvested *Staphylococcus aureus* cells were added equivalent to 1 mg. dry wt./ml. to warm broth containing 0·22 μmole Fe/ml. and 0·0025 μc. ^{59}Fe/ml. at zero time. Penicillin (to 0·1 unit/ml.) was added 60 min. later. Samples of 10 ml. were taken at intervals, cooled and the cells washed twice with broth and once with distilled water, so that ^{59}Fe uptake represents irreversibly bound iron. No difference was found when the broth washing was omitted.

Period after inoculation (min.)	Growth (mg. bacterial dry wt./ml. culture)		Bacterial Fe bound (μmole/ml. culture)	
	Control	+penicillin	Control	+penicillin
0	1·0	1·0	0	0
30	1·33	1·33	0·0070	0·0066
61	2·32	2·39	0·0175	0·0148
80	2·68	2·71	0·0245	0·0260
103	3·68	2·85	0·0400	0·0396
122	3·86	2·89	0·0446	0·0418
142	4·12	2·99	0·0526	0·0526
162	4·13	2·86	0·0549	0·0549
180	4·29	2·91	0·0637	0·0583

Changes in elementary composition

If PBC were itself the 'shuttle' by which a medium constituent is transported into the cell, the entry of this constituent should be rapidly stopped by penicillin above 0·1 unit/ml. If so, the constituent is not glucose (or oxygen) as the oxidation of this substance is unaffected by growth for at least 1 hr. in penicillin (Gale & Paine, 1951) and penicillin is still active anaerobically (see below). It is not an organic substance other than glucose, as penicillin is equally effective on media from which these are missing (Hunter & Baker, 1949; Grelet, 1949). This leaves only the inorganic constituents. Although P uptake is known to be an active function of the osmotic barrier in the staphylococcus used by Mitchell (1953), little is known of the uptake mechanism for K, Na, or Mg. Even so, it would be interesting to see whether the uptake of these metals is affected by penicillin.

Fig. 2 and Table 4 show that the increase of total dry wt. and the uptake of P, K, Na and Mg stopped quite abruptly at intervals after addition of penicillin which did not quite coincide, so that when dry wt. increase had ceased the cells were relatively somewhat deficient in these elements. Usually some loss of these elements started soon after dry wt. ceased to increase.

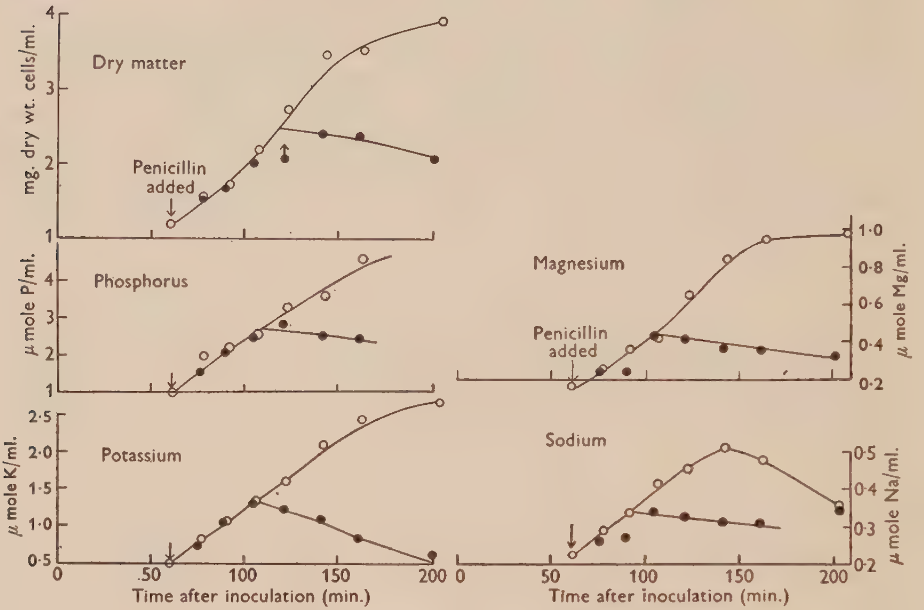

Fig. 2. Changes in elementary composition of *Staphylococcus aureus* growing in presence of penicillin. Conditions as for Fig. 1. Values refer to quantities of element in cells/ml. culture.

Table 3. *Effect of* 0·1 *unit penicillin/ml. on* ^{60}Co *uptake*

Uptake is expressed as μc. bacterial ^{60}Co/l. culture, as accurate assays of Co in cells and medium were not available. Cells contained about 5 μg. Co/g. dry wt., medium about 0·2 μg. Co/ml. To each of four 100 ml. shake-cultures 4 μc. of ^{60}Co were added 65 min. after addition of cells equivalent to 1 mg./dry wt./ml. Penicillin was added to the three test cultures, 60, 75 and 115 min. after inoculation.

	μc. bacterial ^{60}Co/l. culture			
Period after inoculation (min.)	Control	Penicillin added at 60 min.	Penicillin added at 75 min.	Penicillin added at 115 min.
65	0	0	0	0
80	0·326	0·428	0·320	0·326
107	0·376	0·508	0·388	0·376
131	0·356	0·608	0·360	0·424
156	0·400	0·522	0·348	0·408
185	0·396	0·444	—	0·308
208	0·326	0·416	0·292	0·282

'Uptake' of iron was not affected by penicillin, and cobalt 'uptake' was not inhibited but was sometimes stimulated slightly (Tables 2 and 3). The 'uptake' of Fe and of Co measured here differ from the uptakes of P, K, Na and Mg in that they include any equilibration between cell and medium. Ca, rather surprisingly, was present in the washed cells in amounts too small to measure accurately by the method used.

Table 4 also shows the mean elementary composition of the staphylococcal cells during logarithmic growth. The values for P and K were fairly constant over the growth period in the control cells, but the Na content rose to a peak during most active growth and fell towards the end of the logarithmic phase. The Fe and Mg content increased by approximately 70 % of the initial value. Allowing for incomplete packing, the cells probably occupy about 5 ml./g. of the cell-pad volume of 7·5 ml./g. It is interesting that the cells contain more P, K, Fe and Co per unit volume than the medium, but less Na, Mg and Ca (unless some is lost in the washing process).

Changes in phosphate distribution

Fig. 2 shows that the net gain of PO_4 from the medium was not affected by penicillin until just before the increase in dry matter ceased. Fig. 3 shows that the accumulation of P in the PhI and PhO stopped about the same time as, or earlier than, the assimilation of total P. In no experiment was there any sign of the PhO (nucleotide?) fraction increasing above that of control. However, net synthesis of the large molecule P (polyglycerophosphate complex (PGP) + deoxyribonucleic acid (DNA) + ribonucleic acid (RNA) + lipid phosphate) continued at the expense of the PhO, but at a slower rate. This suggests that one or more component has ceased to be formed, possibly PGP (Mitchell & Moyle, 1950) as no accumulation of nucleotides suggesting inhibition of nucleic acid synthesis was found. Very little P was lost from the cells until about 90 min. after uptake ceased. Fig. 3 suggests that the cessation of P uptake is due to an interference with the transport mechanism, as otherwise a considerable and lasting accumulation of P in the PhI would be expected when uptake ceased. The alternative possibility, that PO_4 continues to enter the cell's inorganic phosphate pool but in a loosely bound form which was completely washed out between harvesting and assay, is unlikely as Fig. 3 shows that there is still a considerable pool of PhI and PhO phosphates which are not washed out.

Comparison of the rates of entry of ^{32}P-labelled phosphate into the various fractions (expressed as relative specific activities, or 'R' values, in Table 5) shows that the specific activity of the total P stayed constant at the value occurring as the time uptake ceased, so that there was no exchange between cell and medium after this time. Thus, the active uptake mechanism did not continue simultaneously with an equivalent loss from say, PGP, where P is not known definitely to enter via the inorganic pool. Nevertheless, there were marked rearrangements in the internal distribution of P. The R value of the PhI rose more rapidly than the control initially, then dropped to very low values, while those of the PhO and large molecule P were practically constant for several hours. Mitchell & Moyle (1953) said that it is not known whether the lipid phosphate and PGP are derived from the phosphate of the medium (mI) or the shuttle compound which transports the phosphate across the

Table 4. *Elementary composition of* Staphylococcus aureus *during logarithmic growth*

Samples were taken between 60 and 200 min. after addition of 1 mg. dry wt. washed cells/ml. warm broth. Two values in a space indicate the results from two identical experiments, one of which is shown in Fig. 2.

	P	K	Mg	Na	Ca (approx.)	Fe	Co (approx.)
μmole/g. dry wt. in control (mean 60–200 min.)	1094, 1131	722, 605	200, 218	114, 164	20	8·3	0·1
μmole/g. dry wt. in control at time of stoppage of dry-matter accumulation in penicillin-treated culture	1132, 1189	677, 633	—, 228	153, 178	—	8·3	—
μmole/g. dry wt. in penicillin-treated culture, at time of stoppage of dry-matter accumulation	990, 1087	632, 520	—, 173	90, 134	—	8·3	—
Interval in min. between adding penicillin and cessation of uptake (±5 min.)*	38, 50	37, 47	—, 45	24, 32	—	>160	—
Atoms/cell (approx.) taking $3·5\times10^{12}$ cells/g.	3×10^8	$1·1\times10^8$	6×10^7	3×10^7	3×10^6	2×10^6	3×10^4
μmole/ml. wet cells taking 5 ml./g. (see text)	200	120	40	23	4	1·5	0·017
μmole/ml. medium	10·5	15·7	124	100	70	0·24	0·004

* The times for inhibition of increase of dry matter were 54 and 57 min.

Table 5. *The effect of penicillin on the relative specific activities* (R) *and on total* P *in various phosphate fractions*

Penicillin (0·1 unit/ml.) added 6 min. after inoculation of *Staphylococcus aureus* equivalent to 0·2 mg. dry wt./ml. warm broth containing ^{32}P. Paired values in the body of the table represent R: μmole bacterial P/ml. culture.

	Phosphate fraction							
Time	PhI		PhO		PGP+DNA+RNA+LP		Total P	
(hr.)	Control	+penicillin	Control	+penicillin	Control	+penicillin	Control	+penicillin
0	0 : 0·030	0 : 0·030	0 : 0·060	0 : 0·060	0 : 0·16	0 : 0·016	0 : 0·25	0 : 0·25
1·0	0·69 : 0·070	1·0 : 0·045	0·29 : 0·153	0·24 : 0·118	0·60 : 0·37	0·43 : 0·29	0·48 : 0·63	0·42 : 0·51
2·33	1·1 : 0·275	0·17 : 0·057	0·62 : 0·602	0·26 : 0·103	1·0 : 1·30	0·40 : 0·43	0·94 : 2·23	0·34 : 0·57
3·1	1·0 : 0·870	0·39 : 0·038	0·68 : 0·310	0·33 : 0·052	0·98 : 2·80	0·50 : 0·35	0·90 : 4·00	0·43 : 0·55
5·4	1·0 : 0·730	0·17 : 0·050	1·0 : 0·235	0·60 : 0·040	1·2 : 3·35	0·35 : 0·35	1·1 : 4·66	0·31 : 0·50

osmotic barrier without turnover, or from AI with turnover. The lack of exchange of ^{32}P between cell and medium after net uptake was stopped by penicillin is evidence that either (*a*) significant PGP synthesis directly from mI has also ceased by the time the P transport has stopped, or (*b*) all P enters via the one transport mechanism, and P for PGP comes via AI with turnover, i.e. reversibly. The lack of effect of penicillin on the P uptake until 50–60 min. after the addition of penicillin suggests that the P for PGP (comprising nearly 40 % of the total cell P, Mitchell & Moyle, 1951*b*) cannot be derived directly from the medium, and at the same time cease to enter PGP much before the total P uptake ceases. This means that the statement of Mitchell & Moyle (1950) that the PGP content falls very rapidly after addition of penicillin is also evidence for (*b*) above. The changes in specific activity shown in Table 5

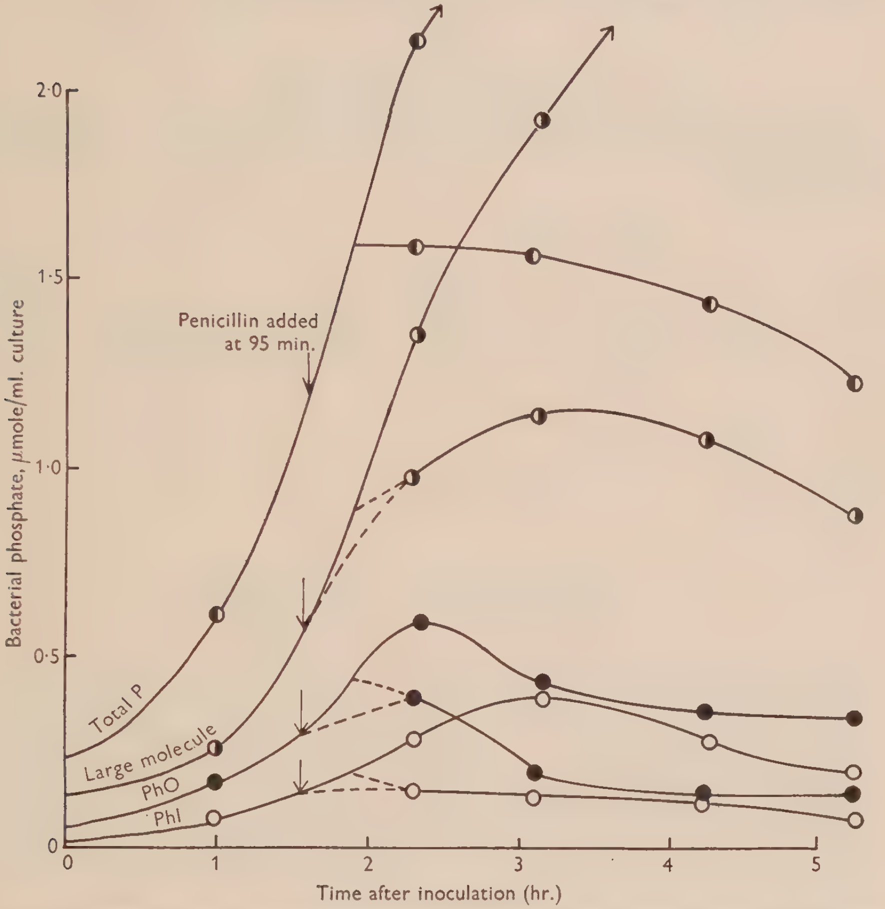

Fig. 3. Changes in the distribution of phosphate between the various fractions of *Staphylococcus aureus* on growth in presence of penicillin. Cells equivalent to 0·2 mg. dry wt./ml. were added to warm shaken broth and penicillin to 0·1 unit/ml. was added 95 min. later. (○—○)=PhI, (●—●)=PhO, (◑—◑)='large molecule phosphate', (◐—◐)=total phosphate. In each case the upper curve is the control.

suggest that the synthesis of some component(s) of PGP + DNA + RNA + LP, presumably PGP, is inhibited within 60 min. by penicillin, but the reverse loss back into the PhI is still allowed. Table 6 shows that synthesis of total lipid P continued after accumulation of dry matter had ceased. Growth in 0·1 unit penicillin/ml. for 1 hr. did not affect the proportion by weight of the 'lipid particle' fraction sedimenting between 5000 and 12,000 *g*.

Table 6. *Effect of penicillin on lipid phosphorus of growing* Staphylococcus aureus *cells*

Fifty ml. of overnight culture were added to 300 ml. of warm broth in each of 14 Roux bottles, and penicillin (0·06 unit/ml.) was added to 7 bottles 0·66 hr. after inoculation. The contents of one control and one penicillin-treated bottle were harvested at different times during incubation at 36°, washed twice and assayed for lipid P.

	Control culture			Penicillin-treated culture		
Time after inoculation (hr.)	Viable count orgs./ml. culture × 10^6	Dry wt. cells, µg./ml. culture	Bacterial lipid P, µmole PO_4/l. culture	Viable count orgs./ml. culture × 10^6	Dry wt. cells, µg./ml. culture	Bacterial lipid P, µmole PO_4/l. culture
0·8	2·2	10	0·83	2·2	8·6	0·81
1·9	8·8	48·7	1·32	7·1	26·2	1·47
2·8	16	68·4	1·71	1	26·2	0·73
3·9	38	87·7	2·02	0·4	20·7	1·43
5·0	46	119	2·26	0·3	23·1	1·59
5·9	62	135	2·84	0·4	17·6	0·65
6·9	78	186	3·35	0·4	16·5	0·66

Sensitivity of Staphylococcus aureus *to penicillin under anaerobic growth conditions*

Three drops of broth containing 5, 300 and 15,000 viable cells respectively were placed on each of a duplicate series of agar plates containing 0·2, 0·1, 0·05, 0·025, 0·012, 0·06, 0·03 and 0 unit penicillin/ml. One series was incubated aerobically and the other anaerobically in a McIntosh and Fildes jar at 37°. Although growth was slower and the final size of colony was smaller with anaerobic growth, there was no difference in the number of colonies after 16 and 64 hr. which appeared at each concentration whether grown with or without air. Thus the initial site of penicillin action cannot be concerned with the metabolism of oxygen as such.

Increase of bound penicillin during growth

Provided that no growth occurs in the presence of penicillin, the penicillin-binding capacity of cells is the same whether they are harvested during the logarithmic phase of growth (Fig. 4) or during the stationary phase, or washed with distilled water and suspended in neutral buffer, namely 0·9 ± 0·1 unit/g. dry wt. However, when the cells were grown in the presence of penicillin the uptake progressively increased to approximately double after about 30 min. Previous experiments (Cooper, 1954) showed that resting cells contained a reserve of

PBC, liberated by rupture, which is approximately equal to the PBC available to penicillin in the intact cell. Thus such cells actually contain twice as much PBC as they appear to in the intact state. From Fig. 4, allowing for this reserve PBC, the total rate of synthesis of PBC in the control culture is about 2·5 units/l. of culture/100 min., whereas the rate of exposure of PBC during the first 30 min. after addition of penicillin is about 10·2 units/l. of culture/100 min. Therefore, either the rate of synthesis is greatly stimulated by penicillin, or else the 50 % of the total PBC which is held in reserve is rapidly mobilized and exposed to penicillin, while synthesis continues at the normal rate. The latter view is rendered more likely by the finding that, when cells were grown for 60 min. in presence of 0·1 unit non-radioactive penicillin/ml., washed three times with distilled water and ruptured, the radiopenicillin-binding capacity of the lipid particles was decreased from 12 units/g. (control) to 1·8 units/g. Contact of the intact cells with 0·1 unit non-radioactive penicillin/ml. without growth only decreased the uptake to 6 units/g. Thus, at least 70 % of the reserve PBC had been exposed to penicillin during growth, the remaining small uptake by the lipid particles possibly being due to some PBC synthesized during this period of growth and not yet exposed to penicillin. The reserve PBC can be rapidly exposed by mechanical rupture at 2° in presence of 1 % formalin when enzymic synthesis would be much slowed, so that exposure without synthesis seems quite possible. Some synthesis of PBC may continue for a time after exposure of the reserve PBC is largely complete

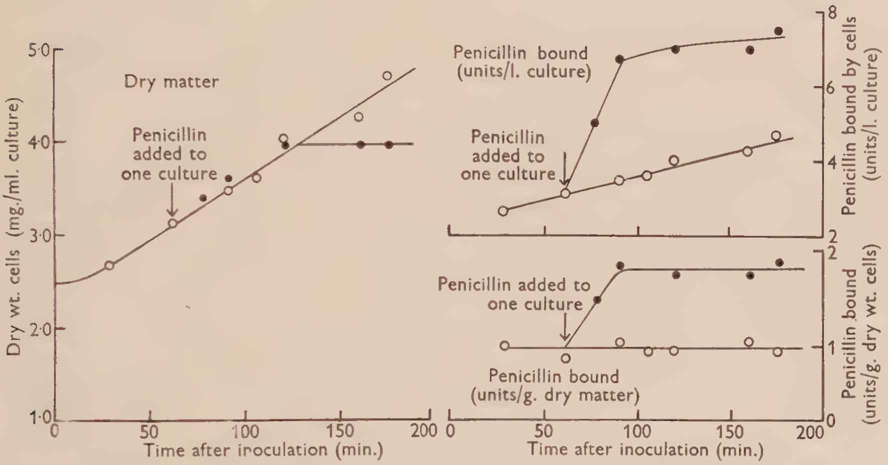

Fig. 4. **Penicillin binding by *Staphylococcus aureus* in the growing phase. Cells added equivalent to 2·5 mg. dry wt./ml. (○—○) = control culture, samples (10 ml.) of the culture taken at intervals were mixed with cold radiopenicillin to give 0·1 unit penicillin/ml. Growth was stopped within 1 min. by cooling. (●—●) = penicillin-treated culture, radiopenicillin added to the culture to 0·1 unit./ml at 60 min. Growth was allowed to proceed in presence of penicillin, samples being taken at intervals and rapidly cooled. Both sets of samples were then washed before ^{35}S assay and measurement of dry wt. cells/ml.**

(Fig. 4). Eagle (1954) was unable to find any significant effect of the metabolic state of bacteria on their ability to bind penicillin, but the increase in uptake on growth found by Cooper & Rowley (1949) and by Few, Cooper & Rowley (1952), was also observed by Maass & Johnson (1949*b*).

DISCUSSION

So far from the osmotic barrier of Gram-positive bacteria being merely a semipermeable dialyzing membrane which retains large but not small molecules, it is impermeable to phosphate (Mitchell, 1953) while allowing free entry of lysine, (Gale, 1947). The cell is, therefore, obliged to assimilate phosphate by transport mechanisms requiring energy. Little is yet known of the bacterial uptake mechanisms for other elements or water. It may not be sufficient, however, to assume that all positive ions enter merely by diffusion as 'gegenions', as certain authors have invoked active mechanisms for K^+ transport into erythrocytes (e.g. Ponder, 1950; Harris & Maizels, 1952). Also sheep kidney cortex mitochondria may assimilate Mg^{++}, PO_4''', K^+ and Na^+ by processes which require energy (e.g. Bartley & Davies, 1954).

Effects of penicillin on properties of the osmotic barrier. The association of PBC with cell-wall lipoprotein suggests that penicillin may initially disorganize some function of the osmotic barrier (Cooper, 1954). The glutamate assimilation is unaffected until about 60 min. after addition of penicillin (Gale & Taylor, 1947) when it is suddenly very seriously impaired, but it is not at present certain whether, as seemed likely earlier, the uptake of glutamate is in fact by active transport mechanisms (Gale, 1953). The present work shows that at least one active mechanism, namely phosphate assimilation, abruptly ceases to function at about the same time, even if its inactivation is not irreversible. It might also be inferred from the sudden stoppage of increase of dry matter soon after, while the cell can still probably synthesize peptides or proteins, deoxyribonucleic acid and ribonucleotides from internal resources (Gale & Taylor, 1947; Hotchkiss, 1950; Gale & Paine, 1951; Gros & Macheboeuf, 1953; Mitchell & Moyle, 1951*a*; present work), that the replication of the bulk of cell material (carbon and nitrogen compounds) is blocked at the stage of taking into the cell. The time course of the impairment of K^+, Na^+ and Mg^{++} uptake closely resembles that of dry matter and P, but it remains to be seen whether these metals enter by active or passive transport mechanisms. If passive, then uptake cessation is due to prevention of synthesis or uptake of their several receptors (not peptides, certain large molecule phosphates, or glucose oxidation products), and is not a direct result of impairment of osmotic barrier function. Nevertheless, whether or not these changes represent a general impairment of the active functions of the osmotic barrier, it is evident that they take some time to appear, so that they probably represent secondary effects of penicillin. Certain properties such as retention of solutes, and possibly exclusion of water, are also not affected until somewhat later, so that breakage of the osmotic barrier is not an early effect of penicillin either. This is possibly reflected in the unaffected synthesis of lipid phosphates and the lipid particle fraction when the organism is grown in the presence of penicillin.

Later secondary effects of penicillin. Meanwhile, respiration, fermentation and oxidation of glucose, and lysine uptake (Gale & Taylor, 1947; Gros & Macheboeuf, 1953) continue with peptide and nucleic acid synthesis, although the nucleoide-oxidizing ability is considerably impaired by the time that turbidity reaches its peak. In view of the probable drop in internal pH value due to depletion of alkali metals while respiration continues and the increase in osmotic permeability, it is in fact surprising that more enzyme deficiencies have not been noticed, particularly those involving Mg and P. As the glutamate assimilation system appears to require Mg or Mn for its energy supply (Gale, 1949), the deficiency of Mg may be the cause of its impaired function. Also K^+ stimulates uptake of glutamic acid by *Staphylococcus aureus* (Davies, Folkes, Gale & Bigger, 1953).

Deficiency of certain elements may also account for another effect of penicillin. The cellular acid-soluble ribonucleotides increase very markedly on growth with penicillin, while RNA remains steady (Park, 1952*a*–*c*, 0·1 unit penicillin/ml.; Mitchell & Moyle, 1951*a*, 1 μg. penicillin/ml.) and this could be interpreted as a block in RNA synthesis at the stage of nucleotide polymerization. Synthesis of DNA is apparently unaffected. The present work shows that the use of 3 % phenol to liberate the small molecular components of the cell did not reveal any accumulation of soluble nucleotides after addition of penicillin. On the contrary, large molecule P synthesis continued at the expense of the pool of phenol-soluble organic phosphorus. It is, therefore, suggested that penicillin allows ribonucleotides to enter RNA normally for a time, but the cell's elementary deficiencies somehow weaken this RNA synthesis, so that the more recently added ribonucleotides are removed by the rather drastic treatment with trichloroacetic acid. The RNA isolated from Gram-positive cells contained about 5 % firmly bound Mg (Henry & Stacey, 1946) and Mg is essential in considerable amounts for Gram-positive cells (Webb, 1951). Absence of Mg decreases the rate of RNA synthesis to one-tenth in *Escherichia coli* (Abelson & Aldous, 1950), but it is not known at present what role if any metallic elements play in nucleic acid structure. It is interesting that the giant forms induced by deficiency of Mg (Webb, 1951) are similar to those formed in sub-lethal penicillin concentrations (Fleming, Voureka, Kramer & Hughes, 1950), although it is true that other substances besides penicillin can induce giant forms.

The nature of the reaction controlled by PBC. The exact nature of this reaction is still not known; none of the characters examined was damaged very soon after adding 0·1 unit penicillin/ml. It is tempting to speculate that the increase of bound penicillin caused only by growth in the presence of penicillin is the result of the forced substitution of the normal substrate of PBC by penicillin. Thus the obscured half of the total PBC may be progressively diverted by penicillin from duties which involve it in a routine cycle, and the rate of PBC release (above that due to its normal synthesis) would represent the normal rate of turnover of PBC when metabolizing its substrate. If so, it can be calculated from this rate and from the rate of increase of dry matter (Fig. 4) that the 'free' PBC turns over about ten times in order to double the cell mass. Each cell will bind 300 molecules of penicillin, and if 1 molecule of penicillin displaces 1 molecule of PBC substrate, then in order to produce two cells from one, PBC would metabolize 3000 molecules of substrate—the amount of substrate required by one cell. Several assumptions are involved here, but even if this rough calculation is ten times too low, then a quantitative change in a reaction of such a small size would still require a very specific analytical method to detect it.

On the bases of (1) the effectiveness of penicillin in the absence of all organic matter other than glucose, and in the absence of air, and (2) the likelihood that above 0·1 unit penicillin/ml. the use of PBC is completely stopped either immediately penicillin is added (as all free PBC is bound within 2–3 min. or at least by the time of the earliest observed abnormality—i.e. block in Na uptake, 20–30 min.), it is possible to say what are not functions of PBC, namely: gross assimilation of oxygen, Na, Mg, K, P, Fe, Co, or material contributing largely to the dry matter, including all organic substances; synthesis of the major part of lipid phosphates and the lipid particle fraction. The very small turnover rate of PBC deduced above would also exclude uptake of Ca, and synthesis of the major part of fractions present in large amount such as PGP, DNA, RNA, cell wall material and proteins, although synthesis of a minor component of these fractions would be possible but difficult to detect. It is also possible that PBC is the shuttle mechanism for the intake of a trace element required in amounts similar to those of cobalt, although if this be so the shuttle is not actuated by thermal agitation as is that for phosphate (Mitchell, 1953), since no turnover of PBC occurs in the resting state.

In conclusion, it may be said that although penicillin is bound by a component very close to the osmotic barrier and most of the earliest large-scale changes have some connexion with active properties of the cell wall, yet these changes do not begin with this organism until at least 20–30 min. after addition of penicillin under conditions where the initial lesion should be evident at once. It remains to be seen what is the function of the system initially affected by penicillin, and how this small defect causes damage to certain cell wall activities while other metabolic activities continue.

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The Action of Fumagillin on a Bacteriophage of *Staphylococcus aureus*

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SUMMARY: Concentrations of fumagillin which allowed host growth prevented multiplication of phage 209 on *Staphylococcus aureus* 209. Fumagillin had no action on free phage and little on the adsorption of phage to its host, but when added 15 min. or less after the start of the latent period it prevented phage multiplication. Fumagillin present during adsorption was diluted out during the latent period; the longer fumagillin was present the smaller was the phage yield. Prolonged exposure of the host + phage mixture in Ringer's solution to a concentration of fumagillin too low to prevent phage multiplication lengthened the latent period and decreased the yield of phage, whether fumagillin was present during the burst or not. Under these conditions, when sufficient fumagillin was added to produce a phage-inhibitory concentration and later diluted out, the yield of phage was constant when the dilution was made in the period 0–20 min.

Asheshov, Strelitz & Hall (1949) reported the isolation of an *Aspergillus* species which produced two antibiotics active against bacteriophages, one being specific for 'streptophages', the other for some staphylococcal phages. These antibiotics did not act on free phage, or affect adsorption of phage to host. Even when added immediately after infection, the antibiotics did not always prevent a burst. When added 10–15 min. before the start of the second cycle of growth they prevented the second burst. The antibiotic active against staphylococcal phages was later shown (Asheshov, Strelitz & Hall, 1952) to be identical with the antiphage antibiotic, fumagillin, isolated by Eble & Hanson (1951) from a strain of *Aspergillus fumigatus*. As part of a programme of research into virus chemotherapy, the work of Asheshov *et al.* (1949) on the mode of action of fumagillin has been extended, using the system *Staphylococcus aureus* strain 209 + phage 209. The results are reported here.

EXPERIMENTAL

Host. Staphylococcus aureus strain 209 was used as the bacterial host.

Phage. Stock phage 209 of titre $6{\cdot}8 \times 10^7$ particles/ml. was prepared as a bacteriologically sterile suspension in 50 % glycerol. It was stored at 5° as a 1/100 dilution in Ringer's solution.

Media. Lab-Lemco broth was used throughout as growth medium. Ringer's solution was used when a fluid was required which did not support host growth.

Fumagillin. This was obtained through the courtesy of Dr R. Coghill, Abbotts Laboratories Inc., U.S.A. It was made up as a 0·1 % (w/v) suspension in 10 % (v/v) acetone in water and stored at −20°.

Titration of phage. For one-step growth experiments: to 1 ml. phage suspension in a test tube, 1 ml. of a 24 hr. culture of *Staphylococcus aureus* 209 and 3 ml. nutrient (tryptic digest) agar were added. After the contents had been mixed, the tubes were sloped and incubated at 37° overnight before reading. In all other experiments the mixture was poured on 15 ml. nutrient agar base.

Methods. These were essentially those of Mills (1953). In all one-step growth experiments, host cells previously suspended for 2 hr. in Ringer's solution (Benzer, 1952) were used for adsorption. Times were always measured from the moment of dilution in broth. All work was carried out at 37°. In one-step growth experiments phage liberation occurred over a long period; because of this, measurements of phage yield were made before liberation had ended (usually at 120 min.). Yields were only compared under conditions where the latent period was constant, and as sampling and counting phage was always complete in less than 5 min., it is believed that little error was introduced.

RESULTS

All points on graphs are the mean of three counts. The latent period of phage 209 on *Staphylococcus aureus* 209 was about 60 min. (Fig. 3). The yield of phage in one-step growth experiments was usually four to six times phage input. No estimate was made of the percentage adsorption.

Active concentration of fumagillin. In a serial dilution test (Chantrill *et al.* 1952) fumagillin at 16 μg./ml. allowed host growth but at 0·6 μg./ml. prevented phage growth. In one-step growth experiments it prevented phage growth at 0·5 μg./ml. but not at 0·25 μg./ml. Except where otherwise stated, 1 μg./ml. fumagillin was used for this work.

Action of fumagillin on host growth. Fumagillin at 1 μg./ml. had no observable effect on host growth during the first 4 hr. as measured by poured plate colony counts. After this time, growth was slightly retarded (Fig. 1).

Action on free phage. When phage 209 was exposed to 2 μg. fumagillin/ml. in broth at 37° for 3 hr., the titre remained constant at $1{\cdot}5 \times 10^5$ particles/ml.

Table 1. *The action of fumagillin* (1 *μg./ml.*) *on the adsorption of phage* 209 *on* Staphylococcus aureus 209 *in Ringer's solution at* 37°

Fumagillin		Count (plaques/tube)	
During adsorption	During phage growth	10 min.	120 min.
−	−	12, 11, 12	77, 63, 64
−	+	7, 7, 6	12, 12, 4
+	−	17, 13, 11	42, 43, 37
+	+	8, 6, 13	7, 12, 9

Action on adsorption. When fumagillin was present during adsorption and diluted out at the start of the latent period, a burst occurred but the yield of phage was a little smaller (Table 1); hence any action fumagillin had on adsorption could only be small.

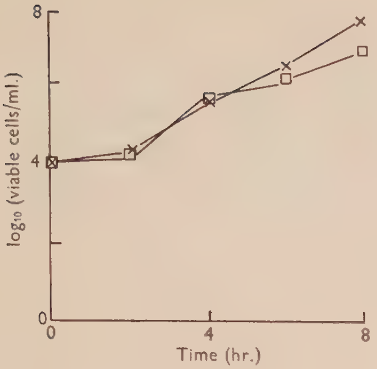

Fig. 1

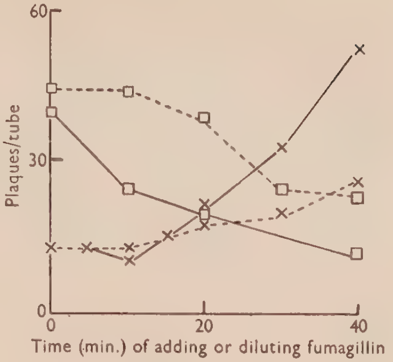

Fig. 2

Fig. 1. The action of fumagillin (1 μg./ml.) on the growth of *Staphylococcus aureus* 209 in broth at 37°. ×—×, —fumagillin; □—□, +fumagillin. Growth was estimated by poured-plate colony counts.

Fig. 2. The effect of fumagillin (1 μg./ml.) on the yield of phage 209 on *Staphylococcus aureus* 209 in one-step growth experiments. Squares, fumagillin initially present at 1 μg./ml., diluted to 0·1 μg./ml. at different times; crosses, fumagillin added to 1 μg./ml. at different times. Dotted lines, preliminary exposure of host and phage to 0·1 μg./ml. fumagillin for 90 min. before transfer to broth. Full lines, no preliminary exposure to fumagillin. Counts were made by sloping 1 ml. samples and counting plaques after incubation overnight at 37°.

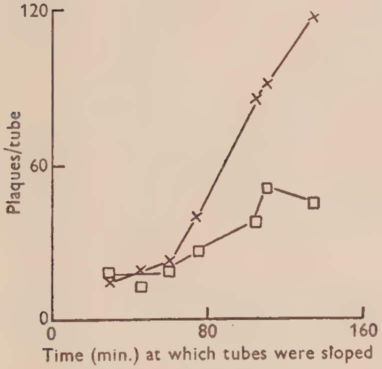

Fig. 3

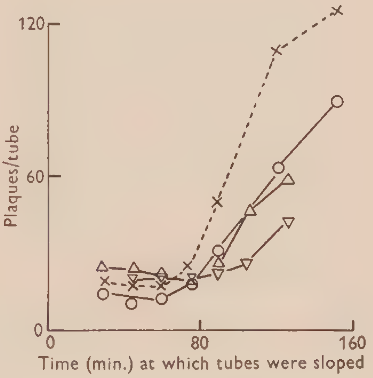

Fig. 4

Fig. 3. The effect of adding fumagillin (1 μg./ml.) at 30 min. on the latent period of phage 209 on *Staphylococcus aureus* 209 in broth. ×—×, —fumagillin; □—□, +fumagillin. Counts were made by sloping 1 ml. samples and counting plaques after incubation overnight at 37°.

Fig. 4. The effect of diluting fumagillin from 1 μg./ml. to lower concentrations on the latent period of phage 209 on *Staphylococcus aureus* 209 in broth. × - - - ×, control—fumagillin; ○—○, fumagillin diluted to 0·002 μg./ml. at 0 min.; △—△, fumagillin diluted to 0·1 μg./ml. at 0 min.; ▽—▽, fumagillin diluted to 0·1 μg./ml. at 40 min. Except in the control, fumagillin was present during adsorption at a concentration of 1 μg./ml. Counts were made by sloping 1 ml. samples and counting plaques after incubation overnight at 37°.

Action on intracellular phage

Time of action. Adsorption was carried out in the absence of fumagillin which was added at various times after dilution into broth. There was no burst when fumagillin was added at 15 min., but a small burst when it was added at 20 min. (Fig. 2). This behaviour differed from that reported by Asheshov *et al.* (1949), who found that fumagillin did not prevent a burst even if added when phage and host were mixed. When fumagillin was added at 30 min., the latent period was not affected (Fig. 3).

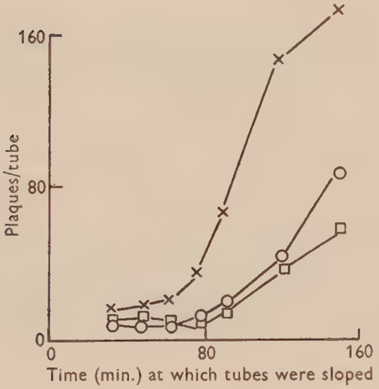

Fig. 5

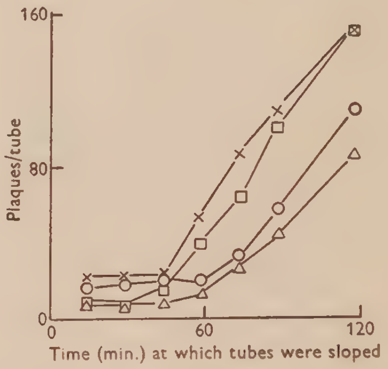

Fig. 6

Fig. 5. Effect of preliminary exposure to fumagillin (0·1 μg./ml.) on the latent period of 209 phage on *Staphylococcus aureus* 209 in broth. ×—×, control—fumagillin throughout; ○—○, fumagillin absent during phage growth; □—□, fumagillin (0·1 μg./ml.) present during phage growth. After adsorption of phage to host, the mixture was held at 37° in Ringer's solution for 1½ hr. in the presence or absence of fumagillin.

Fig. 6. The action of fumagillin (0·1 μg./ml.) on the liberation of intracellular 209 phage from *Staphylococcus aureus* 209 by cyanide. ×—×, control, —fumagillin—cyanide; ○—○, +fumagillin — cyanide; □—□, — fumagillin + cyanide; △—△, + fumagillin + cyanide. KCN was added to a final concentration of 0·03 M at the times shown. At 150 min. it was diluted 1:100 and 1 ml. samples were then counted. When KCN was omitted, samples were diluted 1:100 at the times shown, and 1 ml. samples counted immediately.

Effect of diluting out. Fumagillin was present during adsorption and after transfer to broth. At various times during the latent period a dilution was made to a fumagillin concentration of 0·1 μg./ml., and this concentration was not altered for the rest of the experiment. The later the suspension containing fumagillin was diluted the greater was the effect on phage yield (Fig. 2): the latent period was increased by about 25 min. irrespective of the time of dilution (Fig. 4); this increase was found to be due to the residual fumagillin. When the fumagillin was diluted to 0·002 μg./ml. immediately after adsorption, the latent period was not affected (Fig. 4).

Effect of exposure to fumagillin at 0·1 μg./ml. After adsorption in the absence of fumagillin, the host + phage mixture was diluted in Ringer's solution containing fumagillin 0·1 μg./ml. The mixture was held at 37° for 90 min. and then diluted in broth. Under these conditions, the latent period was increased and the phage yield decreased (Fig. 5). The presence of 0·1 μg. fumagillin/ml. during the actual burst had no further effect on the latent period, but resulted in a still smaller yield of phage (Fig. 5).

Preliminary exposure to 0·1 μg. fumagillin/ml. in the above manner, did not affect the time of action of fumagillin (1 μg./ml.) added at different times during the latent period (Fig. 2). When, however, fumagillin was added to 1 μg./ml. at the start of phage growth, and subsequently diluted to 0·1 μg./ml., for the period 0–20 min., the yield of phage was independent of time of dilution but decreased when the dilution was made at 30 min. (Fig. 2).

Effect of fumagillin on the appearance of intracellular phage. Fumagillin was present at a concentration of 1 μg./ml. during adsorption and was diluted to 0·1 μg./ml. on transfer to broth. At various times samples were taken and cyanide added to a final concentration of 0·03 M to liberate any intracellular phage particles (Doermann, 1951). Each mixture was left for 150 min., diluted 1/100 and 1 ml. samples sloped. An increase in phage titre was found only when cyanide was added 15 min. before the end of the latent period (i.e. at 60 min.) or at times later than this. In the absence of fumagillin, intracellular phage was also detected 15 min. before the end of the normal latent period (i.e. at 45 min., Fig. 6). Under the conditions used, cyanide had no action on free phage 209.

DISCUSSION

Asheshov *et al.* (1949) found that their antibiotic had no action on free phage, on host growth or on the phage adsorption on its host. They also found that their antibiotic did not always prevent a burst even when added at the time of mixing phage and host. In the present work fumagillin prevented phage multiplication when added during the first 15 min. of phage growth. This discrepancy might be due to differences in a number of factors: experimental conditions, phage and host used, purity of the antibiotic, or even the antibiotic itself as it is not clear which of their two Asheshov *et al.* (1949) were using.

From the results reported above, fumagillin appears to have at least two different actions on the *Staphylococcus aureus* 209 + phage 209 system. At low concentrations fumagillin lengthens the latent period and decreases the yield of phage; at high concentrations fumagillin (when added early enough) has a true virustatic action, and (when added late enough to allow some phage growth) no action on the latent period. It seems reasonable to assume that when fumagillin is initially present at 1 μg./ml. and later diluted out, the decreased yield of phage with increased time of dilution reflects both the 'latent period effect', which is only slowly established, and the virustatic effect. The experiments in which fumagillin was always present at a concentration of at least 0·1 μg./ml. were attempts to study the virustatic effect by itself. The results are consistent with the hypothesis that fumagillin interferes with a stage in the development of vegetative phage. This stage occurs about 20 min. after the start of phage growth. As the first detectable intracellular phage is not found till about 15 min. before the end of the latent period (i.e. at 45 min. in the absence of fumagillin and at 60 min. in 0·1 μg. fumagillin/ml.) the action of fumagillin, unlike that of proflavine on T2 phage of *Escherichia coli* B (Foster, 1948), is not on phage maturation. Neither does it act on the stages immediately after infection, as does a phenanthridine on some phages of *Pseudomonas aeruginosa* (Mills, 1953; Dickinson, 1954). The work reported here does not allow any hypothesis about the nature of the stage in phage synthesis inhibited, but the specificity of action of fumagillin (Asheshov *et al.* 1949) suggests that the stage may be one peculiar to a few staphylococcal phages.

I wish to thank Dr Löis Dickinson for her advice and criticism in this work, Mr C. E. Coulthard for his interest in it, and Miss M. L. Rose for technical assistance.

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Transamination Reactions in *Saccharomyces fragilis*

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SUMMARY: A dialysed cell-free extract of *Saccharomyces fragilis* Jörgensen was found to catalyse the transfer of the amino group from alanine, aspartic acid, isoleucine, leucine, norleucine, methionine, ornithine, phenylalanine, tryptophan, tyrosine or valine to α-ketoglutaric acid with the production of glutamic acid and the corresponding keto acid. The transaminase reactions leading to the formation of alanine, aspartic acid, phenylalanine or tryptophan from glutamic acid and the corresponding keto acid were also catalysed by the yeast extract. The addition of pyridoxal phosphate to the incubation mixture increased the rate of transamination between α-ketoglutaric acid and any one of the following amino donors: aspartic acid, leucine, methionine, phenylalanine, tryptophan or tyrosine.

The rate of formation of glutamic acid from α-ketoglutaric acid and leucine was most rapid at pH 8, but the rate of amino transfer from aspartic acid did not vary with pH over the range 6·0–9·0. The equilibrium constant for the aspartic–glutamic transamination reaction was found to be 2·2 at pH 7·8 and 37°.

The transfer of the amino group from aspartic acid or leucine to α-ketoglutaric acid was not inhibited by any one of five antibiotics tested, but was partially inhibited by 10^{-3} M-semicarbazide.

Since the discovery of transamination reactions by Braunstein & Kritzmann (1937) opinion concerning the range and importance of such reactions in biological systems has undergone a cyclic change. The early studies by these two workers indicated that many amino acids were capable of transferring their amino groups to an acceptor such as α-ketoglutarate. Later work by Cohen (1939, 1942) seemed to restrict transaminase activity to two reactions involving glutamic and aspartic acids in one case and glutamic acid and alanine in the other. For several years transaminase reactions were considered to play only a limited role in the formation and interconversion of amino acids. Then Roine (1947) demonstrated that a direct transfer of the amino groups of valine, leucine, and isoleucine to α-ketoglutaric acid was catalysed by extracts of *Torulopsis utilis*, and Feldman & Gunsalus (1950) showed that dried cell preparations of *Escherichia coli*, *Pseudomonas fluorescens* and *Bacillus subtilis* catalysed the formation of glutamate from α-ketoglutarate and any one of thirteen amino acids, the amino acids utilizable varying slightly with the organism. Cammarata & Cohen (1950) showed that twenty-two amino acids other than aspartic acid, glutamic acid and alanine can participate in transamination in pig heart, liver and kidney tissues. Hird & Rowsell (1950) demonstrated that similar transaminase enzymes are present in rat liver mitochondria, and that additional enzymes (Rowsell, 1951) are present which utilize keto acids other than α-ketoglutarate as amino acceptors. Fincham (1951) demonstrated that dialysed extracts of *Neurospora crassa* catalysed some ten transamination reactions.

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Studies on the concentration of aspartic and glutamic acids within the cell by *Saccharomyces fragilis* Jörgensen suggested that transaminase reactions might be involved in the internal metabolism of these dicarboxylic acids. The present work was therefore undertaken to determine the range of transaminase systems in *S. fragilis* and their relative importance in the metabolism of this organism.

METHODS

Organism and enzyme preparations. Saccharomyces fragilis Jörgensen from the Centraalbureau voor Schimmelcultures, Baarn, was used for the preparation of transaminase extracts. The yeast was grown in approximately 150 ml. of liquid medium in Roux bottles for 17 hr. at 25°. The medium consisted of (%, w/v): $NH_4H_2PO_4$, 0·1; $(NH_4)_2HPO_4$, 0·1; KH_2PO_4, 0·2; Marmite, 0·01; 1% (v/v) of a trace element solution (20·0 g. $MgSO_4.7H_2O$; 1·0 g. NaCl; 0·5 g. $FeSO_4.7H_2O$; 0·5 g. $MnSO_4.3H_2O$; 0·05 g. $CuSO_4.5H_2O$; 10 ml. 0·1 N-H_2SO_4; per litre of solution). Glucose was sterilized separately and was added to the autoclaved medium to a final concentration of 4% (w/v). After incubation the cells were harvested, washed once with 0·066 M-KH_2PO_4 and once with distilled water. In the earlier experiments the washed cells were treated with 10 vols. of cold (4°) acetone, washed with acetone and ether and the powder stored in a desiccator over H_2SO_4 or $CaCl_2$. The acetone powder (50 mg.) was rubbed up with 0·1 M-phosphate buffer (pH 7·8; 1·0 ml.) and the extract dialysed with stirring against 0·02 M-KCl at 1–2° for 6–8 hr. The debris was removed by centrifugation at 1000 *g* and the clear supernatant fluid tested for transaminase activity. Storage at −15° preserved the activity of the extracts for 1–2 weeks.

In the later part of this work, a Hughes press (Hughes, 1951) was used for the preparation of the cell-free extracts. Alumina powder (McIlwain, 1948) was mixed with an equal (wet) weight of yeast and the mixture crushed in a press previously cooled to −15°. The resulting material was extracted with 0·05 M-phosphate buffer (pH 7·8; 5 ml./g. wet wt. cells) and the extract dialysed and centrifuged as before.

Assay for transaminase activity. The formation of glutamic acid was demonstrated qualitatively by paper chromatography. Whatman no. 3 paper was spotted with 1–2 μl. of test solution and the chromatogram developed with butanol/water/acetic acid (5:4:1) for 8–15 hr. by the descending technique (Consden, Gordon & Martin, 1944). In some instances, where the amino acids under study had widely different R_F values, ascending chromatograms were developed for 2 hr. with phenol saturated with water (Hird & Rowsell, 1950).

Glutamic acid and aspartic acid were estimated manometrically by the methods of Gale (1945) and Krebs (1950) respectively.

In qualitative work, incubations were made in 15 ml. centrifuge tubes containing 0·3 ml. enzyme solution, 10 μmole L-amino acid (20 μmole if the DL-isomer was used), 10 μmole α-ketoglutarate and 0·5 ml. 0·1 M-phosphate buffer (pH 7·8) containing approximately 20 μg. pyridoxal phosphate/ml., in a total volume of 1·0 ml. Three controls were included in each set of experiments: one without amino acid, one without keto acid, and one with enzyme and buffer only, the amino acid and keto acid solution being replaced by 0·1 ml. 0·1 M-phosphate buffer (pH 7·8). The tubes were flushed with nitrogen and closed with rubber stoppers. Incubation was at 37° for 1, 3 or 16 hr. The reaction was stopped by placing the tubes in boiling water for 3 min. The precipitate was centrifuged down and the clear supernatant used for a chromatogram.

For quantitative experiments, enzyme and buffer containing pyridoxal phosphate as above were placed in the centre compartment of a Warburg vessel and amino acid and/or keto acid in the side bulb. After gassing with nitrogen and equilibrating at 37° the contents were mixed. At the end of the incubation the pH was adjusted to 4·5 with N-HCl and the remaining traces of reaction mixture washed from the side bulb into the centre compartment with sufficient 0·2 M-acetate buffer (pH 4·5) to bring the volume to 2·5 ml. The vessels were then placed in boiling water for 3 min. to inactivate the enzyme. After cooling, 0·5 ml. of a suspension of *Clostridium welchii* was placed in the side bulb and the glutamic acid estimated manometrically.

When aspartic acid was to be determined, the incubation was carried out in the same manner but the inactivation was modified by using 0·1 M-acetate buffer (pH 5·0) to bring the volume of the reaction mixture to 2·0 ml. The suspension of *Clostridium welchii* was placed in the main compartment of the Warburg vessel so that any glutamic acid present in the sample was decarboxylated during the equilibration period. The other components of the aspartic acid test, i.e. 0·1 ml. α-ketoglutarate, 0·2 ml. partially purified horse heart transaminase and 0·05 ml. freshly distilled aniline were placed in the side bulb. The determination then followed the method of Krebs (1950).

RESULTS

Survey by chromatography of possible amino donors

Feldman & Gunsalus (1950) used paper chromatography in their studies on the transaminase systems of *Escherichia coli*, *Pseudomonas fluorescens* and *Bacillus subtilis*. Difficulties arose when a similar technique was applied to work with dried cells or crude extracts of *Saccharomyces fragilis* since this yeast contains a variety of amino acids in the free state within the cell, with especially high concentrations of glutamic acid and alanine. Any extract of these cells contains amounts of glutamic acid which interfere in the interpretation of a qualitative method such as chromatography. By growing the organism in a simple ammonium salt medium (Taylor, 1947) the concentration of glutamic acid within the cell was decreased but was still too high for clearly defined results. However, dialysis against 0·02 M-KCl for 6–8 hr. was found to decrease the glutamic acid content of the extract below the level required to give a colour with ninhydrin. A further difficulty was encountered when it was found that appreciable amounts of glutamate were produced in mixtures containing α-ketoglutarate but no amino acid. The extract was shown to contain a glutamic dehydrogenase capable of reducing 1·35 μmole triphosphopyridine nucleotide/ml. extract/2·5 min., which probably accounts for glutamate formation from α-ketoglutarate in the absence of added amino donors. This activity could be greatly diminished, and in some cases eliminated, by further dialysis of the extract for 24 hr., presumably by removal of the triphosphopyridine nucleotide. Other control mixtures in which (*a*) α-ketoglutarate was omitted, (*b*) the enzyme preparation was heated to 100°, (*c*) α-ketoglutarate was replaced by oxalacetate or pyruvate, showed no trace of glutamate formation.

Twenty-two amino acids were tested both qualitatively and quantitatively for ability to transfer their amino group to α-ketoglutarate in the presence of the dialysed yeast extract. A spot corresponding to glutamic acid was formed when aspartic acid, leucine, phenylalanine, methionine, tryptophan or tyrosine was the amino donor.

Quantitative estimation of transamination

The results of the quantitative experiments in which the glutamate formed was measured manometrically are given in Table 1. The experiments were carried out with extracts of acetone powders and with extracts of whole cells prepared with the Hughes press.

Acetone powder extracts. After incubation for 1 hr., aspartic acid, leucine, phenylalanine, methionine, tyrosine, tryptophan, isoleucine and valine (in order of decreasing activity) were found to act as amino donors with α-ketoglutarate as amino acceptor. After 3 hr. quantitative, but not qualitative, differences were observed. It was possible, however, by increasing the time of reaction to 16 hr. to confirm the presence of the two transaminases of much lower activity which had isoleucine and valine as substrates. In some preparations there seemed to be some evidence of slight alanine, ornithine, threonine and arginine transaminase activities but the results were variable.

Hughes press extracts. The absence of an alanine-glutamate transaminase from the acetone powder extracts was unexpected. It seemed possible that the acetone treatment received by the cells was too severe and inactivated the enzyme which catalysed this reaction. Therefore the survey was repeated using an extract prepared from whole cells crushed in the Hughes press at −15° (Table 1). In addition to the eight amino acids given above, alanine, norleucine and ornithine were able to transfer their amino groups. In general the activities observed with Hughes press extracts were higher than with extracts of acetone powders.

Effect of pyridoxal phosphate on rate of transamination

Chromatographic experiments showed that the amount of glutamate formed by transamination from leucine, aspartic acid, phenylalanine, methionine, tyrosine or tryptophan was increased in the presence of pyridoxal

Table 1. *Transaminase activity in extracts of* Saccharomyces fragilis

1 ml. of incubation mixture contained: 10 μmole amino acid (L-isomer), 10 μmole α-ketoglutarate, 0·3 ml. dialysed yeast extract, 0·1 M-phosphate buffer (pH 7·8) containing 20 μg. pyridoxal phosphate/ml. Incubation was at 37° in N_2 and glutamate was estimated on boiled, deproteinized mixtures. All figures are corrected for glutamate formed (<0·5 μmole/mg. extract N) in the absence of an added amino donor.

	μmole glutamate/mg. extract N			
	Acetone powder extract		Hughes press extract	
Amino acid	1 hr.	16 hr.	1 hr.	16 hr.
L-Alanine	0	0	5·35	10·29
L-Arginine	0	0	0	0
L-Aspartic acid	5·65	25·14	8·25	15·15
L-Citrulline	0	0	—	—
L-Cysteine	0	0	—	—
L-Cystine	0	0	—	—
Glycine	0	0	—	0
L-Histidine	0	0	—	—
L-Hydroxyproline	0	0	—	—
L-Norleucine	—	—	—	12·02
L-Isoleucine	0·6	2·11	—	12·88
L-Leucine	5·67	16·84	11·39	17·15
L-Lysine	0	0	—	0
L-Methionine	2·79	10·2	4·51	10·6
L-Ornithine	0	0·85	—	12·86
L-Phenylalanine	3·67	11·0	2·24	9·84
L-Proline	0	0	—	—
DL-Serine	0	0	—	0
DL-Threonine	0	0	—	0
L-Tryptophan	1·73	6·39	4·39	9·19
L-Tyrosine	2·03	7·3	2·87	9·44
L-Valine	0·52	3·89	—	11·97

phosphate. That the degree of activation is not the same in each system is seen from the quantitative data for aspartic acid, leucine and tryptophan (Table 2). Tryptophan gave the greatest response, no glutamate being formed in the absence of pyridoxal phosphate. Aspartic acid showed the smallest increase in activity, while the leucine system showed an intermediate response. Partial resolution of the transaminase(s) into apo-enzyme and coenzyme during preparation of the extract could account for this increase in activity in the presence of pyridoxal phosphate.

Table 2. *Effect of pyridoxal phosphate on rate of transamination*

1 ml. of incubation mixture contained: 10 μmole amino acid, 10 μmole α-ketoglutarate, 0·1 M-phosphate buffer (pH 7·8), 0·3 ml. dialysed yeast extract, 20 μg. pyridoxal phosphate as indicated. Incubation was at 37° for 1 hr. in N_2.

	μmole glutamate/hr./mg. extract N	
Amino acid	Without pyridoxal phosphate	With pyridoxal phosphate
L-Aspartic acid	4·82	6·84
L-Leucine	2·23	4·82
L-Tryptophan	0	1·88

Glutamic acid as an amino donor in transaminase reactions

Evidence for the reversibility of four of the transamination reactions, aspartic-, alanine-, phenylalanine- and tryptophan-glutamic acid, was obtained chromatographically (Fig. 1). Only these four reactions, for which the equivalent keto acids were readily available, were tested. In each case, material corresponding to the amino acid was formed on incubation of the Hughes press extract with glutamic acid and the keto acid.

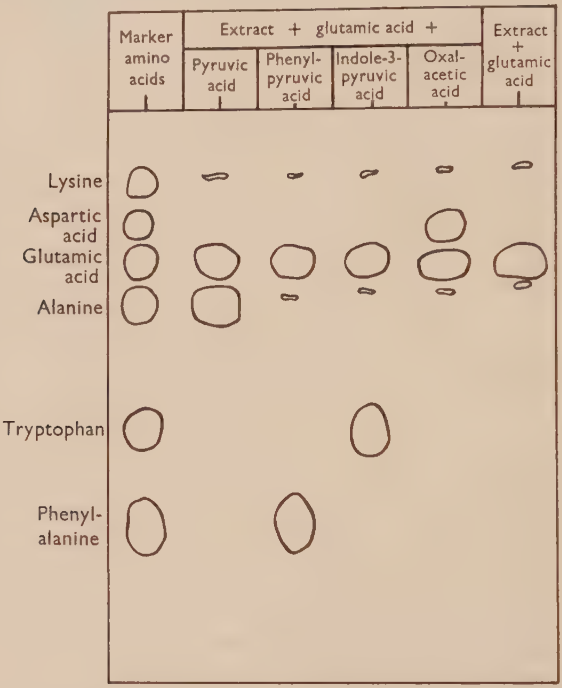
Marker amino acids
Extract + glutamic acid +
Pyruvic acid
Phenyl-pyruvic acid
Indole-3-pyruvic acid
Oxal-acetic acid
Extract + glutamic acid
Lysine
Aspartic acid
Glutamic acid
Alanine
Tryptophan
Phenyl-alanine

Fig. 1. Diagrammatic representation of paper chromatogram showing formation of alanine, phenylalanine, tryptophan and aspartic acid from glutamic acid and the corresponding keto acid catalysed by *Saccharomyces fragilis* extract. One ml. of incubation mixture contained: 10 μmole glutamic acid, 20 μmole keto acid, 0·3 ml. dialysed Hughes press extract, 20 μg. pyridoxal phosphate, 0·05 M-phosphate buffer (pH 7·8). Incubation was for 16 hr. at 37° in N_2. Reaction stopped by heating at 100° for 3 min. Supernatant fluid used to spot chromatogram. Solvent system: butanol-acetic acid.

Attempts to obtain transamination using oxalacetate or pyruvate as amino acceptor and α-aminobutyrate, leucine, methionine, ornithine, phenylalanine, tryptophan, tyrosine or valine as amino donor were unsuccessful. It would appear that only transaminase reactions in which either α-ketoglutarate or glutamate is a substrate are catalysed by the dialysed yeast extract under the conditions used.

Conditions affecting the α-ketoglutarate-leucine transamination

The two most active transaminase systems, aspartic acid + α-ketoglutarate and leucine + α-ketoglutarate, were studied in more detail.

Rate of glutamate formation. A mixture containing leucine, α-ketoglutarate and dialysed acetone powder extract was incubated and the glutamate formed measured after various intervals. Under the conditions used, the amount of glutamate formed increased continuously during the whole course of the experiment (Fig. 2). Initially there was a rapid rate of transamination which gradually decreased but did not cease. Failure to reach a constant glutamate concentration was probably due to non-enzymic breakdown of the α-keto-isocaproic acid which was formed in the reaction, resulting in a shift of the equilibrium to the glutamate side.

Effect of temperature. The rate of the leucine-α-ketoglutarate reaction was found to increase linearly over the range 25–37° with a temperature coefficient of 1·98. Above 37° the rate of increase was diminished.

Effect of pH. The influence of pH on transamination varies with the source of enzyme and with the particular transaminase reaction studied. Lichstein & Cohen (1945) found a very sharp peak at pH 8·5 for the aspartic-glutamic transaminase activity of intact cells of *Escherichia coli* while the corresponding reaction with dried cells or cell-free extracts of *Streptococcus faecalis* showed little variation over the range pH 6·0–9·5 (Lichstein, Gunsalus & Umbreit, 1945). In extracts of *Torulopsis utilis* the reaction between glutamic acid and oxalacetic acid has a maximum activity at pH 7·8 (Roine, 1947). Fincham (1951) found the amino group transfer from isoleucine, ornithine or phenylalanine to α-ketoglutarate with dialysed neurospora extracts to be most rapid at pH 8, that from aspartate at pH 9 and from alanine at pH 5·5–6·0. Amino group transfer from leucine to α-ketoglutarate by a Hughes press extract of *Saccharomyces fragilis*, tested over a pH range 6·0–9·0, was most rapid at pH 8 (Fig. 3). Neither the formation of glutamate from aspartate + α-ketoglutarate nor the formation of aspartate from glutamate + oxalacetate was sensitive to changes in pH value over the same range. At no point on the curve was there more than a 5 % variation from the mean value.

Effect of antibiotics. The leucine-glutamic acid transaminase and the aspartic-glutamic acid transaminase were not inhibited by aureomycin, chloramphenicol, penicillin, streptomycin or terramycin at 100 μg./ml. Both reactions were, however, partially inhibited by 10^{-3}M-semicarbazide, the former system by about 60 % and the latter by about 45 %.

Reversibility of the aspartic-glutamic acid transaminase system

There is chromatographic evidence for the reversibility of this reaction (Fig. 1); the equilibrium has been measured quantitatively by approaching it from both sides (Fig. 4). The initial rate of reaction A (glutamic acid → aspartic acid) is much more rapid than that of reaction B (aspartic acid → glutamic acid), an observation consistent with the results of Cohen (1940) who used animal tissues and of Roine (1947) who used a *Torulopsis utilis* extract. Curve A shows a maximum formation of aspartic acid after 2 hr. followed by a slight decrease, probably due to the instability of oxalacetic acid at the pH value of the experiment. In the case of the reverse reaction (B) the spontaneous decomposition of oxalacetic acid results in an increase in the amount

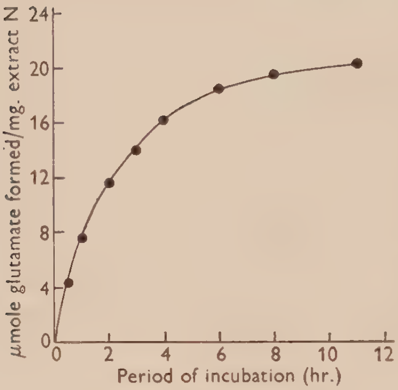

Fig. 2

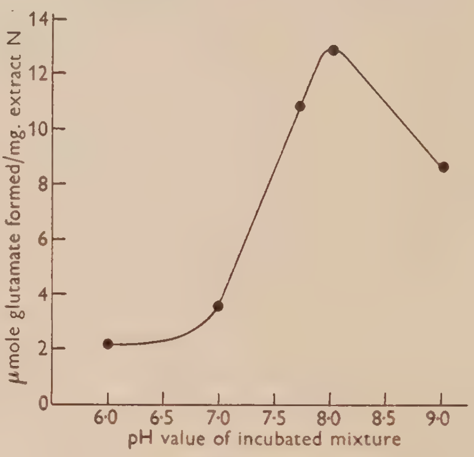

Fig. 3

Fig. 2. Rate of glutamate formation from leucine + α-ketoglutarate. One ml. of incubation mixture contained: 10 μmole L-leucine, 10 μmole α-ketoglutarate, 0·2 ml. dialysed yeast extract, 0·1 M-phosphate buffer (pH 7·8) containing 20 μg. pyridoxal phosphate/ml. Incubated at 37° in N_2.

Fig. 3. Effect of pH value on glutamate formation from leucine + α-ketoglutarate. Incubation mixture as in Fig. 2 except for variation in pH value as indicated. Incubated at 37° in N_2 for 2 hr.

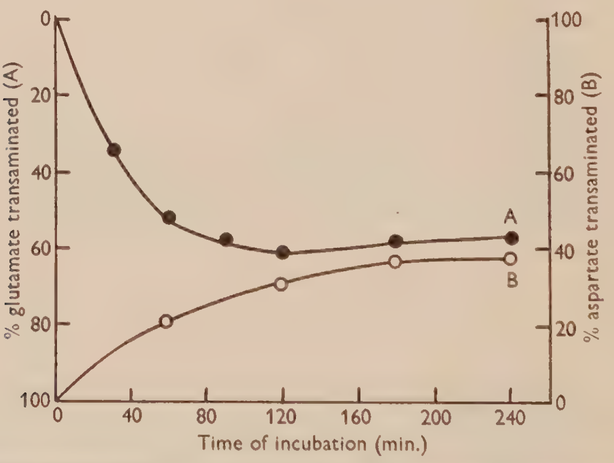

Fig. 4. Transamination between (A) glutamate and oxalacetate, (B) aspartate and α-ketoglutarate. One ml. of incubation mixture contained: 7 μmole L-amino acid, 14 μmole keto acid, 0·2 ml. yeast extract, 0·1 M-phosphate buffer (pH 7·8) containing 20 μg. pyridoxal phosphate/ml. Incubated at 37° in N_2.

of glutamate formed. Calculation of the equilibrium constant for the reaction gives a value of

$$K=\frac{[\text{aspartate}]\,[\alpha\text{-ketoglutarate}]}{[\text{glutamate}]\,[\text{oxalacetate}]}=2{\cdot}2 \text{ at pH } 7{\cdot}8 \text{ and } 37^\circ,$$

a figure which is in reasonable agreement with that of 3·5 obtained by Cohen (1940) with purified pig heart enzyme.

I wish to thank Dr R. Davies and Dr E. F. Gale, F.R.S., for their interest in this work. I also wish to express my appreciation to the U.S. State Department for a Fulbright Fellowship during part of this investigation, and to the National Institute of Microbiology of the U.S. Public Health Service for a Predoctoral Fellowship during the remaining part. I am grateful to Dr R. E. Kallio, Dr E. V. Rowsell and Dr W. W. Umbreit for their gifts of indole-3-pyruvic acid, phenylpyruvic acid and pyridoxal phosphate, respectively.

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The Sporulation of *Bacillus sphaericus* stimulated by Association with other Bacteria: an Effect of Carbon Dioxide

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SUMMARY: Sporulation of *Bacillus sphaericus*, NCTC 7582, in a complex medium was strongly stimulated by growth in mixed culture with a sporing strain of *B. cereus* and with sporogenous and asporogenous strains of *B. subtilis*, NCTC 85. There was a similar, but less pronounced, effect with a variety of Gram-positive and Gram-negative organisms. The degree of sporulation of *B. sphaericus* in these mixed cultures depended on (*a*) an external stimulus from the medium and (*b*) the previous cultural history of the *B. sphaericus* inoculum upon which depended its ability to respond to the external stimulus. Sporulation of *B. sphaericus* in pure culture was strongly stimulated by bicarbonate and ketoglutarate, suggesting that increased carbon dioxide production was the stimulating factor in mixed cultures.

The stimulatory effect of one organism upon the growth of another has been observed in bacteria and in fungi (e.g. Grassberger, 1897; Bacon, Burrows & Yates, 1951; Hawker, 1947). In fungi there are examples of stimulation of both growth and sporulation in mixed cultures (Asthana & Hawker, 1936; Hawker, 1939). This report describes a similar phenomenon in the genus *Bacillus* and suggests a mechanism for the effect.

ORGANISMS AND METHODS

A culture of *Bacillus sphaericus*, NCTC 7582, grown for 24 hr. at 37° on casein hydrolysate yeast extract (CCY) agar (Gladstone & Fildes, 1940) was suspended in 50 ml. glucose serum broth (Fry & Greaves, 1951) and freeze-dried in 5 ml. batches. Starting with one of these freeze-dried batches, a series of sporulation tests was made, during which time the organism was maintained by subculturing every 48 hr. on CCY agar, and on potato-extract agar (Robinow, 1951) enriched with 1/10 vol. of CCY medium (Pot. CCY).

The strongly sporing strain of *Bacillus cereus* isolated from air and the sporogenous and asporogenous strains of *Bacillus subtilis*, NCTC 85, were maintained on CCY agar. The other organisms used were subcultured at least twice in Pot. CCY medium before the mixed culture tests.

For the sporulation tests, which were set up in duplicate, 10 ml. batches of liquid Pot. CCY medium in 50 ml. conical flasks were inoculated with 0·5 ml. of a just visibly turbid suspension of organisms and shaken at 37° for periods up to 36 hr.

RESULTS

There was rapid growth and remarkably vigorous sporulation of *Bacillus cereus* and *B. subtilis* in the test medium, which was selected for this reason. The first signs of sporulation appeared at 14 hr., and at 17 hr. almost every cell contained a well-developed spore. *B. sphaericus* taken from the first CCY agar subcultures showed approximately 1–5 % sporulation after 36 hr. At 18 hr. 20–30 % of the cells appeared to have reached a half-way stage of sporulation, but disintegrated before the spore had properly developed. After about twenty subcultures on CCY agar, although growth in the test medium was not diminished, the sporing ability of the organism became almost negligible, until finally no sign of sporulation could be detected. During this time the effect of inoculating test flasks with both *B. cereus* and *B. sphaericus* was also studied. The first *B. sphaericus* subcultures, with some tendency to sporulate when grown alone, showed almost complete sporulation after 18 hr. growth with *B. cereus* (Pl. 1, figs. 1, 2). Later subcultures, which showed no sign of sporulation when grown alone, were stimulated to produce pre-spores, i.e. refractile non-staining areas in the presence of *B. cereus*, and then appeared to disintegrate. After further subcultures on CCY agar, *B. sphaericus* lost its ability to respond to the *B. cereus* stimulus.

When an original freeze-dried stock culture of *Bacillus sphaericus* was maintained by subculture on Pot. CCY agar, there was a marked increase of sporing ability in pure culture. Similarly, cultures which had become asporogenous after repeated subculture on CCY agar, sometimes regained their ability to sporulate after approximately twenty subcultures on Pot. CCY agar. Thus, of four such asporogenous cultures, two regained their original sporing ability completely. The other two remained asporogenous but produced cells containing large granules staining black by the Albert (1921) method and purplish pink with methylene blue and toluidine blue. Some of the granules appeared to be free. Examined in wet preparation, the granules were less refractile than developing spores from which they could readily be distinguished. The phosphorus: nitrogen ratio in washed cells from a 'granular' culture was 0·37 compared with 0·26 in a culture not containing granules. The granules therefore probably contained volutin, i.e. polymetaphosphate (Wiame, 1947).

Sporogenous cultures of *Bacillus sphaericus* grown for 16 hr. in Pot. CCY medium were centrifuged and the cells resuspended in the medium from 9 and 14 hr. cultures of *B. cereus*. After 5 hr. shaking there was only poor sporulation, but large purple-pink granules appeared in most cells (Pl. 1, figs. 4, 5). The appearance of these resuspended cultures was similar to that of the asporogenous granular cultures just described (Pl. 1, fig. 3). After 24 hr. shaking, the resuspended culture consisted almost entirely of non-sporulating cells free from large granules. Similar results were obtained with 10 hr. cultures of *B. sphaericus*.

A slight stimulation of *Bacillus sphaericus* sporulation was observed when the organism was grown in a cellophan tube immersed in a shaken growing culture of *B. cereus*, but this result was not always repeatable.

The effect of growing *Bacillus sphaericus* in mixed culture with other organisms was then studied. Both the sporogenous and asporogenous strains of *B. subtilis* stimulated rapid and almost complete sporulation of the feebly sporing *B. sphaericus*, and increased the 'granulation' of the asporogenous strain. *Staphylococcus aureus* (Oxford strain), *Corynebacterium xerosis*

NCTC 7904, and laboratory strains of *Chromobacterium prodigiosum* (*Serratia marcescens*) and a *Leuconostoc* species had a similar but less pronounced effect. *Escherichia coli*, NCTC 1100, and a laboratory strain of *Pseudomonas aeruginosa* (*pyocyanea*) grew well in mixed culture with *Bacillus sphaericus* but had no apparent effect on its sporulation.

Bacillus sphaericus cells resuspended in culture fluid from a stimulating organism showed very poor sporulation (see above). It appeared therefore that the stimulus in mixed culture was produced during associated growth of the stimulating organism, and was probably due to some non-specific effect such as (i) a more rapid exhaustion of nutrients in mixed culture, or (ii) a reduction of dissolved oxygen or an increase of carbon dioxide concentration in the medium to some critical level. The effect of adding 0·04 M-sodium bicarbonate to the medium as a means of increasing carbon dioxide concentration was next tested. Control flasks were adjusted to the same pH as those to which bicarbonate was added, i.e. pH 8·2. At 18 hr. there was very poor sporulation in the controls, but almost complete sporulation in the presence of bicarbonate (Pl. 1, figs. 6, 7). Final pH values were 9·0 and 9·2 respectively. There was equally strong stimulation of sporulation in the presence of 0·04 M-ketoglutarate (Pl. 1, figs. 8, 9) with initial and final pH values of 7·6 and 8·7 both in the controls and ketoglutarate media. Although the other possibilities mentioned above were not explored, it seemed highly probable that stimulation of *B. sphaericus* sporulation in mixed culture was due to increased carbon dioxide concentration in the medium.

DISCUSSION

Under the conditions described it appeared that the degree of sporulation of *Bacillus sphaericus* depended on (*a*) a stimulus from the medium which could be intensified by growth in mixed culture with a variety of bacteria, and (*b*) an intracellular factor which controlled the ability of the cell to respond to the outside stimulus. Although a high CO_2 concentration in the medium may not have been of prime importance for the initiation of sporulation, its presence appeared to be essential in order to make the process rapid and complete. This may hold for sporing organisms in general, since it has been repeatedly observed that rapid and complete sporulation occurs after a period of vigorous growth, i.e. high CO_2 production. It is specially interesting in this connexion that Cantino (1952) showed that bicarbonate stimulated the production of thick-walled resistant sporangia in the mould Blastocladiella. The function of CO_2 as a sporulation stimulant in the genus *Bacillus* may be connected with its possible stimulatory effect on dipicolinic acid synthesis (Powell, 1953). It seems likely, especially since conversion of lysine to pipecolic acid has been demonstrated (Rothstein & Miller, 1953; Lowy, 1953) that dipicolinic acid is derived from $\alpha\epsilon$-diaminopimelic acid by deamination followed by ring closure and dehydrogenation. It is possible that CO_2, by inhibiting the decarboxylation of $\alpha\epsilon$-diaminopimelic acid (an effect already observed by Hoare & Work, 1954, personal communication), might allow the deamination reaction and subsequent build-up of dipicolinic acid to proceed more rapidly.

In this investigation we obtained an asporogenous culture of *Bacillus sphaericus* by repeated subculture of the organism on a relatively rich medium (CCY). The sporulating power of this culture was in some cases restored by repeated transfer to a less nutritious medium (Pot. CCY). The sensitivity, i.e. the degree of response of a vegetative cell of *B. sphaericus* to the external stimulus, may depend on its ability to divert one or more metabolic pathways in 'abnormal' directions, e.g. for the synthesis of dipicolinic acid, and it is possible that this ability might be enhanced or lowered by adaptive processes on different media, or by variation (Brunstetter & Magoon, 1932; Schmidt, 1950). We found that there was often an increased production of volutin by the asporogenous strain of *B. sphaericus* under conditions which stimulated sporulation of the sporogenous strain. This was not due to the development of acid conditions in the culture (cf. Duguid, Smith & Wilkinson, 1954). It may represent an incomplete response to the external stimulus, i.e. a derangement of metabolism in cells whose metabolic path was blocked as a result of conditions in the medium and could not be diverted in the direction of sporulation. There was also volutin production in sporogenous cells, especially after resuspension in culture medium from a stimulating organism. This may again represent an incomplete response in cells which were capable of sporulation but were receiving an insufficiently sustained stimulus from the medium to drive the sporulation process to completion.

We wish to thank Mrs W. Fryer for technical assistance and Mr R. E. Strange for nitrogen and phosphorus analyses. Acknowledgement is made to the Chief Scientist, Ministry of Supply, for permission to publish this paper.

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EXPLANATION OF PLATE

All films heat-fixed and stained with methylene blue. Magnification ×1500.

Fig. 1. 18 hr. sporogenous *B. sphaericus* in pure culture.
Fig. 2. 18 hr. sporogenous *B. sphaericus* in mixed culture with *B. cereus*.
Fig. 3. 18 hr. asporogenous *B. sphaericus* subcultured repeatedly on Pot. CCY agar, showing granule production in test medium at pH 8·1.
Fig. 4. 21 hr. sporogenous *B. sphaericus*.
Fig. 5. 16 hr. sporogenous *B. sphaericus* resuspended for 5 hr. in *B. cereus* medium.
Fig. 6. 18 hr. sporogenous *B. sphaericus* at pH 9·0.
Fig. 7. 18 hr. sporogenous *B. sphaericus* sporulating with 0·04 M-bicarbonate at pH 9·2.
Fig. 8. 18 hr. sporogenous *B. sphaericus* at pH 8·7.
Fig. 9. 18 hr. sporogenous *B. sphaericus* sporulating with 0·04 M-ketoglutarate at pH 8·7.

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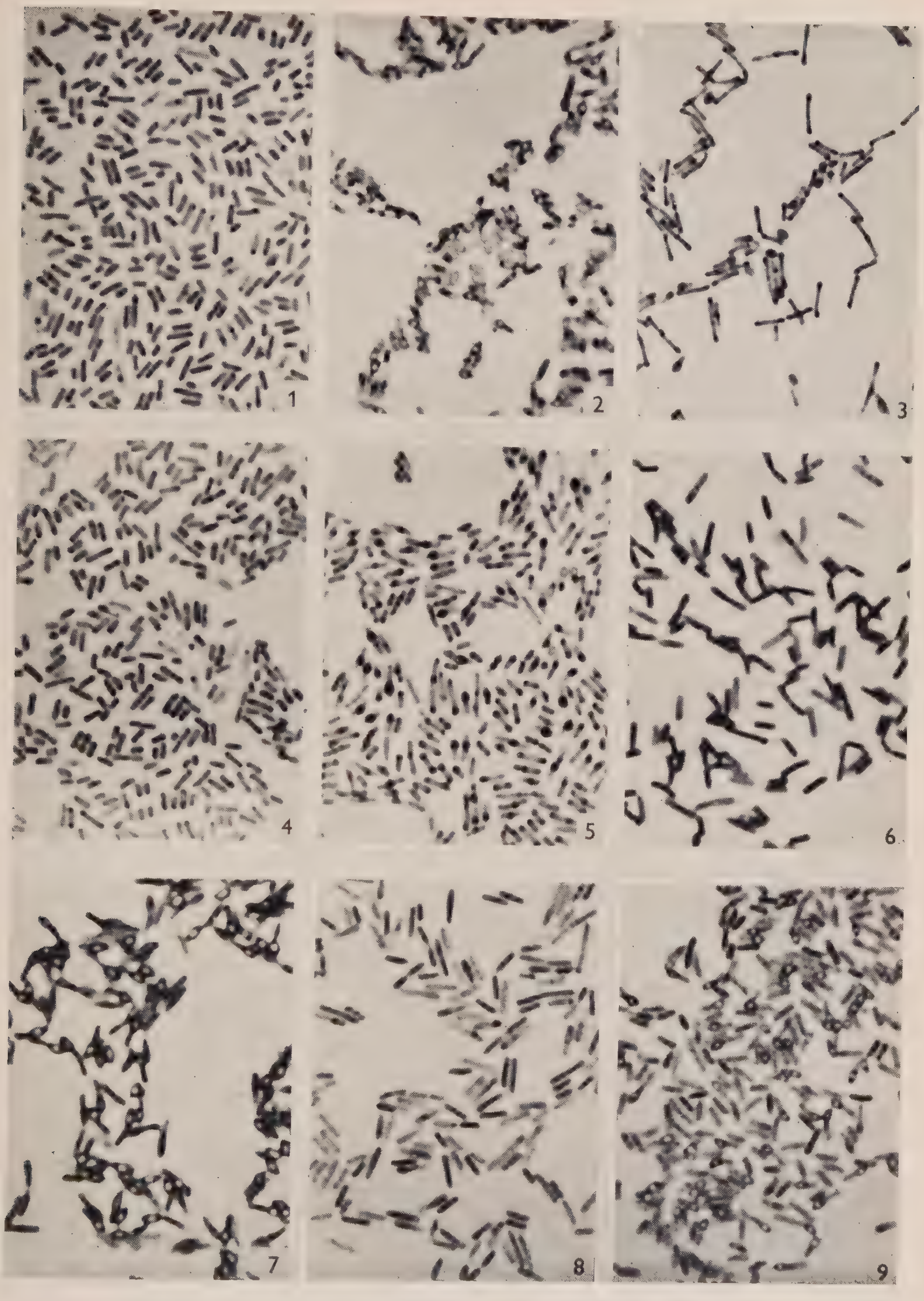

J. F. Powell and J. R. Hunter—Sporulation of *B. sphaericus*. Plate 1.

(Facing p. 58)

Powell, J. F. & Hunter, J. R. (1955). *J. gen. Microbiol.* **13**, 59–67.

Spore Germination in the Genus *Bacillus*: the Modification of Germination Requirements as a Result of Preheating

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SUMMARY: Spore suspensions of *Bacillus megaterium* germinated spontaneously after heat treatment. The extent of germination depended on the degree of close packing of the suspension, the temperature and duration of heating, and on the presence of water. Spontaneous germination was not inhibited by heating with cyanide, azide, 2:4-dinitrophenol or sodium iodoacetate, but was significantly inhibited by sodium fluoride.

Freshly harvested spores of a laboratory strain of *Bacillus cereus* required either inosine or a mixture of alanine + tyrosine + adenosine for optimal germination. After prolonged storage or a short heat treatment, adenosine alone stimulated rapid and complete germination. Heat activation did not occur in the absence of water. It was inhibited to some extent by sodium fluoride. Similar results were obtained with *B. cereus*, NCTC 8035.

After preheating, spores of a virulent and an avirulent strain of *Bacillus anthracis* germinated more rapidly and completely in a mixture of adenosine + tyrosine + alanine, but not in adenosine alone. Inosine stimulated rapid and complete germination of heated suspensions, and was consistently more effective than adenosine. Adenosine deaminase activity has been found in extracts from resting spores of the laboratory *B. cereus* and the avirulent strain of *B. anthracis* but not in *B. megaterium*.

As in previous studies, the criterion of germination was loss of heat resistance accompanied by a change in staining properties, decrease of refractive index and decrease in dry weight (Powell & Strange, 1953). A laboratory strain of *Bacillus megaterium* which required glucose specifically for spore germination was described by Powell (1951). Some freshly harvested spore suspensions of this organism germinated very poorly in glucose solution but were activated by heating at 60° for 30 min. Other suspensions, grown on the same medium germinated completely in glucose solution without heat activation. It was later accidentally discovered that when thick spore suspensions (2×10^{10} spores/ml.) were heated at 60° for 1 to 2 hr., then centrifuged and resuspended in water, spontaneous and complete germination occurred without loss of viability. This behaviour provided a means of obtaining concentrated germination exudate (Powell & Strange, 1953). In suspensions thicker than 1×10^{10} spores/ml. the amount of calcium dipicolinate excreted (Powell, 1953) was actually greater than the saturation concentration, and the substance appeared as a heavy white granular deposit. We have now studied this heat-activated germination in some detail. The modification of germination requirements as a result of preheating has also been studied in two strains of *B. cereus* and in a virulent and an avirulent strain of *B. anthracis*. Freshly harvested, unheated spore suspensions of these organisms germinated in a mixture of L-alanine + tyrosine + adenosine (Hills, 1949).

Several examples of the stimulatory effect of preheating on spore germination in bacteria and fungi have already been described (Goddard, 1939; Hull, 1939; Curran & Evans, 1945; Powell, 1950). The present report describes further examples of this effect, and some attempts to define the stimulation in chemical terms.

ORGANISMS AND METHODS

Washed suspensions of spores of *Bacillus megaterium* were prepared as described previously (Powell, 1951). Spores of the laboratory strain of *B. cereus*, of *B. cereus* NCTC 8035 and of an avirulent strain of *B. anthracis* were obtained after 48 hr. growth at 37° on potato-extract agar (Robinow, 1951) enriched with 1/10 vol. of casein hydrolysate-yeast extract medium CCY (Gladstone & Fildes, 1940). Spores of the virulent strain of *B. anthracis* were obtained by 48 hr. growth on CCY agar. Spore suspensions were washed thoroughly with water at 3° until they were free from cell debris, i.e. at least five times, and stored at 3°. It was generally possible to obtain suspensions containing less than 5 % densely staining, non-viable forms (Powell, 1951) and such suspensions, with the exception of those from the laboratory strain of *B. cereus*, were stable at 3°. Spore suspensions of the laboratory strain of *B. cereus* gradually deteriorated owing to spontaneous germination followed by disintegration and were discarded when the proportion of densely staining forms became greater than 5 %.

All the germination tests were made at 37°, unless otherwise stated, in the presence of 40 mM-phosphate (pH 7·3) at concentrations of 0·5 to $1{\cdot}0 \times 10^9$ spores/ml. The percentage germination was assessed by the staining technique and the speed of germination by repeated measurements of light scattering (Powell, 1950).

For the detection of adenosine deaminase activity, suspensions containing 2×10^{10} spores/ml. in 50 mM-phosphate buffer (pH 7·3) were first broken down mechanically at 3° in a tissue disintegrator (Mickle, 1948) with Ballotini beads (size 12). The enzyme was present in the clear extract obtained by high-speed centrifuging. This extract was incubated for 30 min. with 1 mM-adenosine, and the ultraviolet absorbing constituents were separated using the paper ionophoresis technique of Wade & Morgan (1954). After 2 hr. of ionophoresis the paper was dried and examined in ultraviolet light. Adenosine was separated from the slower-moving inosine by approximately 3 cm. on the paper. Disintegrated spore extracts incubated without adenosine were also examined as controls.

RESULTS

Spontaneous germination of Bacillus megaterium *spores after heat activation*

A suspension of *Bacillus megaterium* spores containing $1{\cdot}5 \times 10^{10}$ spores/ml. was heated in 2 ml. batches for 15, 30, 60, 90 and 120 min. at temperatures between 50° and 78°. After centrifuging for 30 min. at room temperature (20°) the spores were resuspended in 10 ml. of water, allowed to remain at room temperature for 1 hr. and the percentage germination determined by the staining method. When this was plotted against duration of heat treatment a sigmoid curve was obtained for each temperature (Fig. 1). Plotting temperature of activation against the period of heating required to give 50 % germination, a smooth curve (Fig. 2) was obtained which suggested that very long heating periods would be necessary for activation at temperatures lower than 55°, and that there might be a critical temperature of activation. This appeared to be the case: almost complete germination occurred after 18 hr. heating at 45° and practically no germination after 18 hr. at 44°. Different spore crops showed similar but not identical behaviour to that just described. We have the impression that crops grown from cultures which showed strong melanin pigmentation, i.e. a high tyrosinase activity, were considerably more responsive to heat activation. Such crops also had a greater tendency to spontaneous germination during harvesting.

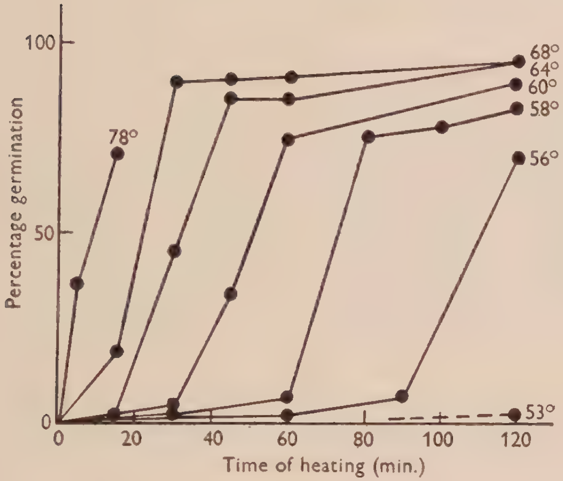

Fig. 1

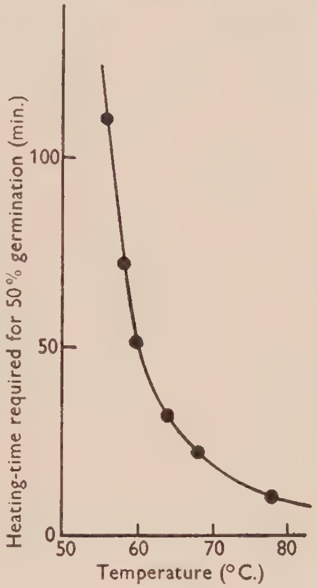

Fig. 2

Fig. 1. Heat-activated spontaneous germination of *B. megaterium* spores.

Fig. 2. Heat-activated spontaneous germination of *B. megaterium* spores. Data from Fig. 1.

During these experiments it was noticed that a white granular deposit of calcium dipicolinate was present in the centrifuged mass of spores before resuspension. It thus seemed that germination proceeded while the spores were packed down, and not during resuspension as was first thought. It was then shown that resuspension was not an important factor in promoting spontaneous germination but that close packing after heat treatment was. Thus a spore suspension heated at 56° for 2 hr. gave only 25 % germination when allowed to stand without centrifuging, and almost complete germination when centrifuged and then simply shaken up. It seemed possible that a comparatively small proportion of spores germinating in the packed cell mass was producing a very concentrated germination exudate which itself had a stimulatory effect on the germination of the remainder. This appeared to be the case. A suspension containing $1\cdot5 \times 10^{10}$ spores/ml. was heated at 56° for 2 hr., then transferred in 2·5 ml. lots to tubes containing 20 or 10 mg. of freeze-dried germination exudate. Germination in these tubes after 1 hr. standing was 61 and 41 % respectively. A control tube of spores alone without addition of germination exudate showed only 18 % germination. When the tubes were kept at 37° for 1 hr. after heat treatment and addition of exudate, the corresponding germination figures were 90 and 82 %, with a control of 36 %. The constituent of the germination exudate responsible for this stimulation has not yet been identified. The isolated exudate fractions, i.e. calcium dipicolinate and the non-dialysable peptide (Strange & Powell, 1954) were tested and found inactive.

As indicated above, a greater degree of germination was obtained by maintaining heat-activated suspensions at 37° than at room temperature (20°). On the other hand, spontaneous germination was completely suppressed by cooling the heated suspension rapidly in ice. No germination occurred during heat treatment, and a suspension could be heated and ice-cooled alternately many times without spontaneous germination occurring. When, finally, the heated suspension was centrifuged and maintained at 37° it germinated completely. It therefore appeared that the heat-sensitive system was activated by high temperatures but could only function between certain limits of temperature.

The heat-activation process was dependent on the presence of available water. Freeze-dried spores were heated at 60° for 2 hr. in a stoppered tube containing P_2O_5, then suspended in water at 37° and centrifuged; no spontaneous germination occurred. Similarly, when freeze-dried spores were heated in glycerol, ethanol, ethylene glycol or a saturated solution of KI, then diluted with water, centrifuged and resuspended, no germination occurred. Control tubes containing freeze-dried spores and water germinated completely after heating and centrifuging.

Spore suspensions were also heated for 2 hr. at 60° in 1 mM-potassium cyanide, 5 mM-sodium azide, 5 mM-2:4-dinitrophenol or 20 mM-sodium iodoacetate, then centrifuged and resuspended in water; no inhibition of heat-activated germination occurred. On heating in 100 mM-sodium fluoride, germination was reduced from 82 % in the control tube to 20 %. In a similar experiment with a different crop of spores, 100 mM-fluoride reduced germination from 80 to 52 %.

Germination of spores of Bacillus cereus *and* Bacillus anthracis

The rate of germination of spores of the laboratory strain of *Bacillus cereus* in tryptic digest broth (TMB), in adenosine + tyrosine + alanine all at 1 mM (AdTAl) and in 1 mM-adenosine was first compared, using the nephelometer method. Germination was generally complete, i.e. >95 % within 5 min. in TMB and in the AdTAl mixture. Different spore crops showed differing behaviour with adenosine. Usually, germination in adenosine was complete in 15 min., but in some cases, only 50 % germination occurred in 1 hr. and this was not increased during a further incubation for 1 hr. The effect of preheating aqueous spore suspensions at 60° for 1 hr. was, in all cases (*a*) to reduce the time required for complete germination in TMB and AdTAl to 2–3 min., and (*b*) to stimulate complete germination in adenosine in 2–5 min. This effect was retained indefinitely.

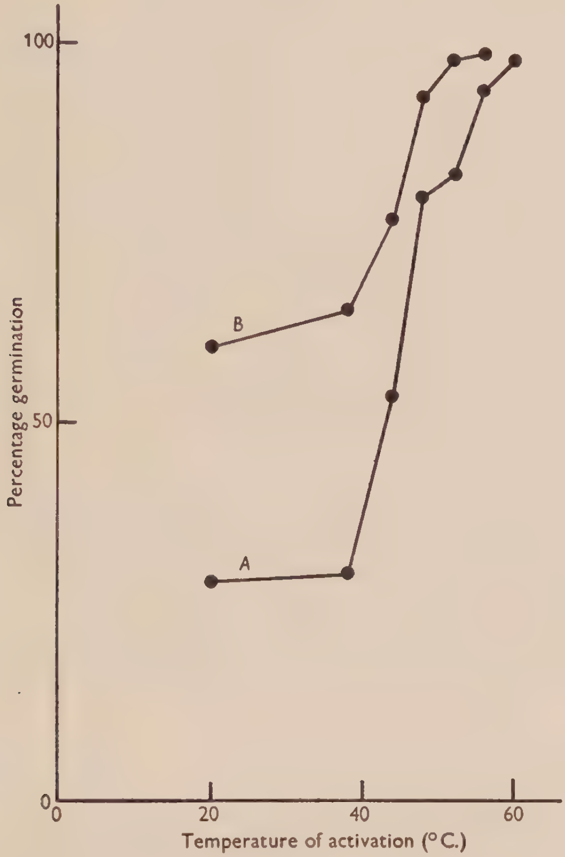

Fig. 3. The effect of 1 hr. preheating on germination of *B. cereus* spores in 1 mM-adenosine at 37°. Percentage germination at A, 10 min.; B, 20 min.

The rate of germination of preheated spores in 1 mM-adenosine was roughly proportional to the temperature of preheating. The effect of heating a spore suspension which germinated slowly in adenosine for 1 hr. at several temperatures between 37 and 60° is illustrated in Fig. 3. Heating for 1 hr. at temperatures below 40° had relatively little stimulatory effect, although germination became more rapid and complete after several weeks storage of the aqueous spore suspension at room temperature or even at 3°. This stimulation appeared not to be due to the liberation of soluble material during heating or storage: heated or stored spores after resuspension in distilled water still germinated rapidly in adenosine alone. The tendency of spores of the laboratory strain of *Bacillus cereus* to germinate and disintegrate slowly on storage has already been mentioned. There was, however, no tendency to spontaneous germination after heat treatment, although the germination exudate from *B. megaterium* had some stimulatory effect on these heated spores.

The stimulatory effect of preheating depended, as with *Bacillus megaterium*, on the presence of water. The rate of germination in 1 mM-adenosine was not increased when dried spores were heated for 1 hr. at 60° in the presence of P_2O_5. The effect of preheating in the presence of 100 mM- and 200 mM-sodium fluoride was also determined. In these tests, the spores were centrifuged after heating then resuspended in adenosine solution. Heat activation was completely suppressed by 200 mM- and partly suppressed by 100 mM-sodium fluoride.

Inosine, guanosine and xanthosine were then tested at 1 mM as germination stimulants. Inosine was found to be more effective than adenosine: with unheated spore suspensions which germinated slowly and incompletely in adenosine (see above) inosine stimulated complete germination within 15 min. Guanosine and xanthosine were considerably less effective than adenosine. They gave less than 20 % germination in 1 hr. when tested alone or in the presence of 1 mM-tyrosine + 1 mM-alanine.

A less complete study was made of the behaviour of *Bacillus cereus*, NCTC 8035, and of a virulent and an avirulent strain of *B. anthracis*. Unheated spore suspensions of *B. cereus*, NCTC 8035, germinated less rapidly in TMB and the AdTAl mixture than those of the laboratory strain, e.g. 60 % in 30 min., 90 % in 1 hr. The substitution of inosine for adenosine in the AdTAl mixture produced complete germination in 30 min. Inosine tested alone did not, as was the case with the laboratory strain, stimulate rapid and complete germination of these unheated suspensions. In the presence of 1 mM-inosine or 1 mM-adenosine there was less than 20 % germination in 1 hr. After heating the aqueous spore suspension for 1 hr. at 60° germination was complete in TMB, AdTAl, InTAl, adenosine and inosine, within 15 min.

Results very similar to those just described for *Bacillus cereus*, NCTC 8035, were obtained with the virulent and the avirulent strain of *B. anthracis*. Inosine at 1 mM stimulated rapid and complete germination of preheated but not of unheated suspensions and was consistently more effective than 1 mM-adenosine when tested in the presence of 1 mM-tyrosine + 1 mM-alanine.

Conversion of adenosine to inosine by spores of Bacillus cereus

Since inosine stimulated rapid germination of unheated spore suspensions of the laboratory strain of *Bacillus cereus* and was consistently more effective than adenosine for spores of *B. cereus*, NCTC 8035, and for the virulent and avirulent strains of *B. anthracis*, it seemed possible that inosine might be in

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fact the germination stimulant, and that germination in a given suspension might depend to some extent on its capacity to convert adenosine to inosine. On this basis, the increased rate of germination in adenosine after heat treatment could be ascribed to activation of an adenosine deaminase system. We therefore attempted to assess adenosine deaminase activity in resting spore suspensions disintegrated before and after preheating.

Tests made with intact spores of *Bacillus cereus* gave results which were difficult to interpret owing to germination occurring during incubation for 30 min. with adenosine. Inosine could not be detected in the medium after paper ionophoresis and examination of the paper in ultraviolet light but two new ultraviolet absorbing spots appeared in positions taken up by adenine and hypoxanthine. These components appeared when germinated spores were incubated with adenosine. It therefore seemed that an extract from disintegrated resting spores would provide a better test system, although disintegration itself might introduce some ambiguity (see below).

Preliminary experiments suggested that disintegrated resting spore suspensions of the laboratory strain of *Bacillus cereus* converted adenosine to inosine and also partly to adenine and hypoxanthine but that there was no correlation between the rate of germination of a given suspension in adenosine and the deaminase activity of the spore extract. Thus, suspensions which germinated very slowly and incompletely in adenosine gave extracts which had a relatively high deaminase activity as crudely assessed by a visual comparison of ultraviolet absorbing spots. Similarly, although preheating greatly increased the germination rate in adenosine, there appeared to be no marked increase of deaminase activity in the spore extract. It was interesting to find that although the enzyme survived heating in the intact spore, it was completely destroyed in the extract by heating at 60° for 15 min. Deaminase activity was also found in spore extracts from the avirulent strain of *B. anthracis* but could not be detected in *B. megaterium* spore extracts.

DISCUSSION

The results obtained with *Bacillus megaterium* spores suggest the possibility that in this organism, at least three systems are operating to produce spontaneous germination after heat treatment:

(i) A system H susceptible to heat-activated reversible hydrolysis $H+H_2O\rightleftharpoons H^W$ with a critical temperature in the region of 45°.

(ii) A system G, which controls germination, depending for its activity on the presence of H^W and functioning within certain limits of temperature not yet closely defined.

(iii) An autocatalytic process, C, due to the stimulatory effect of the germination exudate on heat treated cells.

Thus it might appear that system G does not function at 45°, although after prolonged heating at this temperature H^W is available. Germination does not occur until the temperature falls, but can still be avoided by rapid cooling to 0° at which temperature G is again inactive.

It was interesting to find that the germination exudate itself stimulated the germination of heat-activated spores. Stewart & Halvorson (1953) found alanine racemase activity in resting spores of several species of the genus *Bacillus*, and suggested that the ability of these spores to convert the germination stimulant L-alanine to the inhibitor D-alanine may represent a survival mechanism, i.e. 'a means of ensuring that the supply of viable spores will not all be used up by a single set of favourable circumstances'. An arrangement tending to produce quite the opposite effect appeared to operate in *B. megaterium* under the conditions we have described.

In *Bacillus cereus*, and to some extent in *B. anthracis*, the substitution of inosine for adenosine produced an effect similar to that of heat treatment. It seemed possible, therefore, that an adenosine-deaminase system present in resting spores might be activated by heat treatment, e.g. by a hydrolytic process such as has been suggested for *B. megaterium*. Using a rather crude method of assessment we have so far failed to demonstrate a low deaminase activity in extracts from spores which germinated very slowly and incompletely in adenosine. It may, however, be wrong to draw conclusions about the enzyme activity of intact resting spores from observations on spore extracts. During disintegration, the whole spore structure upon which its special properties depend is destroyed. If, as seems likely, the protoplasm of the resting spore is anhydrous, disintegration would promote hydrolytic processes thus, we suggest, activating the adenosine deaminase system and masking any similar effect due to preheating. The heat stability of the enzyme in the intact resting spore compared with its heat stability in extracts is specially interesting in this connexion and will be more fully investigated.

We wish to thank Mr S. Bailey and Mrs W. Fryer for technical assistance. Acknowledgement is made to the Chief Scientist, Ministry of Supply, for permission to publish this paper.

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Correlation between Morphological and Physiological Characters in the Classification of Members of the Genus *Lactobacillus*

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SUMMARY: Physiologically definable groups within the genus *Lactobacillus* are morphologically distinct. There are marked differences between homofermentative and heterofermentative strains and between physiological groups within the former.

In recent years little use has been made of morphology in the classification of *Lactobacillus*. Much emphasis has been placed upon the pleomorphism of members of the genus, a state of affairs which is almost certainly due to inadequate control of growth conditions (cf. Smith, Gottschall & Wallgren, 1932).

The existence of two main physiological types, homofermentative and heterofermentative is generally recognized, and species within these two types have from time to time been defined, sometimes upon adequate and sometimes upon less adequate criteria. A number of relatively simple tests has been adapted in this laboratory to the purpose of distinguishing certain specific groups, which correspond reasonably closely with those reported by Rogosa, Wiseman, Mitchell, Disraely & Beaman (1953), Sharpe (1955) and Wheater (1955*a*, *b*). These physiological tests will be the subject of a separate communication.

METHODS

The morphology and cytology of 221 strains of *Lactobacillus* were examined. Of these 23 were obtained by courtesy of the National Collection of Industrial Bacteria, Teddington, Middlesex, 3 were isolated from samples of yoghurt, and the remainder from human saliva and carious teeth. All were grown aerobically at 37° in tomato juice broth of the following formula: tinned tomato juice (filtered) 40 % (v/v), Oxoid peptone 1 % (w/v), Difco yeast extract 0·5 % (w/v), sodium acetate 1 % (w/v), glucose 0·5 % (w/v), soluble starch 0·05 % (w/v), Tween 80 0·1 % (v/v), salts A 0·5 % (v/v), salts B 0·5 % (v/v); pH value 6·8. The salt solutions A and B employed were those of Rogosa *et al.* (1953).

The cell-form of the organisms was examined in cultures carried in $\frac{1}{4}$ oz. screw-capped (bijou) bottles of this medium after 24 hr. incubation. Observations were made upon unstained hanging-drop preparations, Gram-stained films, and preparations stained by Hale's (1953) method to demonstrate cell walls.

Colony structure was examined by means of impression preparations (Bisset, 1938). It was found that the following simplified medium, which closely resembles that of Kulp (1927), was best suited to this purpose: tinned tomato juice 40 % (v/v), Oxoid peptone 1 % (w/v), Difco yeast extract 1 % (w/v), glucose 0·5 % (w/v), agar 2·5 % (w/v); pH value 5·5.

Impression preparations were made of 12–24 hr. colonies on this medium. Trials were made with the same medium at pH 7·0, with 5 % (v/v) horse-blood nutrient agar, and with the solid medium obtained by adding 2·5 % (w/v) agar to the more complex tomato juice medium given above. These were unsuitable for the colony impression technique, because the growth tended to be slimy.

RESULTS

The first general observation was that a high proportion of the homofermentative strains possessed a wreathed, 'Medusa-head' colony, whereas heterofermentative strains had 'smooth' structureless colonies.

The heterofermentative group was physiologically variable, but morphologically, remarkably constant. Out of 113 strains, 88 possessed the morphology shown in Pl. 1, fig. 2. The bacilli consisted of single cells or of two cells separated by a cross-wall. Their colonies were 'smooth' and structureless (Pl. 1, fig. 1). In the remainder the bacilli consisted of two or four cells (Pl. 1, fig. 3), but the colony was equally structureless (Pl. 1, fig. 4).

Despite the physiological heterogeneity of this group, it appears that the first morphological type is characteristic of *Lactobacillus fermenti*, whereas that with the longer bacilli (Pl. 1, fig. 3) is found in strains previously classified as *L. brevis* and *L. buchneri*.

Of the 108 homofermentative isolates, 82 were classifiable as belonging to the *Lactobacillus casei-helveticus* group. And of these 56 (68 %) produced the typical 'Medusa-head' colony (Pl. 2, fig. 6). The remainder gave intermediate or, very occasionally, even 'smooth' colonies. The cell form was alike in all cases, however (Pl. 2, fig. 5), and was characteristic. Although superficially similar to the *L. brevis* type in that both have the four-celled structure commonly seen in septate bacilli, the members of the casei-helveticus group were quite distinct from the former in that they appeared much more slender and flexible, including numerous curved cells in pairs (cf. Orla-Jensen, 1919), and also in their formation of sinuous chains. Even those strains which did not produce really typical 'Medusa-head' colonies, usually appeared in chains when observed in hanging-drop preparations.

The second physiological group of homofermentative lactobacilli, the *Lactobacillus acidophilus*, *L. lactis*, *L. bulgaricus* group, corresponds approximately to the genus *Thermobacterium* of Orla-Jensen (1919) and was represented by 14 strains. The colony form in all cases was similar (Pl. 2, fig. 7), being the extreme 'Medusa-head' type, appearing almost as a tangled mass. The cell form was also distinctive, and was seen in its most extreme form in strains corresponding to *Lactobacillus bulgaricus*. The bacilli were very long, with occasional, irregular cross-walls (Pl. 2, fig. 8). The *L. acidophilus* strains varied from this form to chained filaments more like those of the *L. casei-helveticus* type.

Nine strains of *Lactobacillus plantarum* were exceptional among homofermentative types in that their morphology was characteristic of the majority of heterofermenters (Pl. 1, figs. 1, 2).

DISCUSSION

The extremes of morphological type in *Lactobacillus* species are entirely distinct. They vary from short, almost square bacilli in *L. fermenti*, which comprised a very high proportion of heterofermentative strains from saliva, to long filaments with irregular septa in the homofermentative *L. bulgaricus*.

Although the least filamentous of the homofermentative types and the most filamentous of the heterofermentative types may appear almost to converge, their colony forms are for the most part entirely distinct. The 'Medusa-head' colony was so rare as to be almost unknown among the latter, and so common as to be normal among the former, with the exception of one species, *Lactobacillus plantarum*. The colony structure thus revealed in impression preparations is very constant, and does not necessarily accord with the gross appearance of well-grown colonies *in situ*, which is exceedingly variable.

Cytological appearances of the commoner recognizable species of *Lactobacillus* are distinct and provide a valuable taxonomic criterion.

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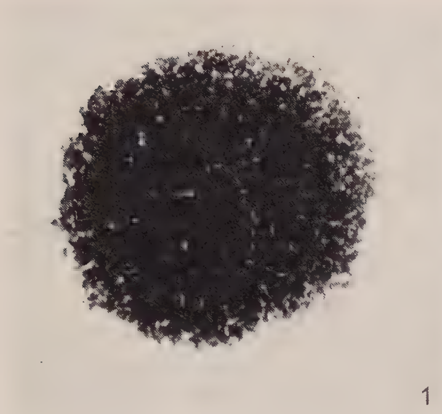

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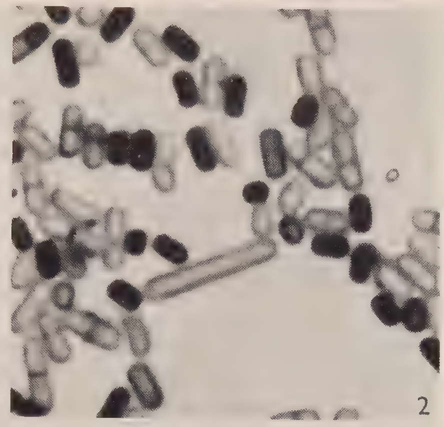

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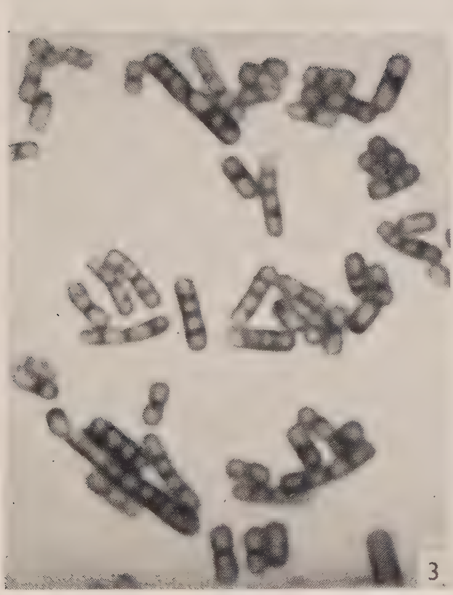

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G. H. G. Davis, K. A. Bisset and C. M. F. Hale—Characters of lactobacilli.
Plate 1

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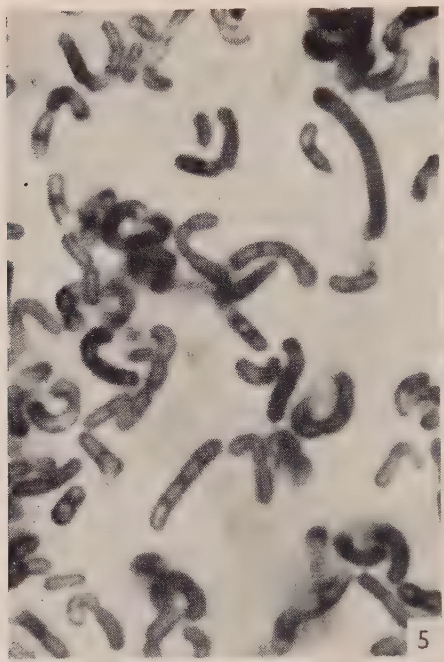
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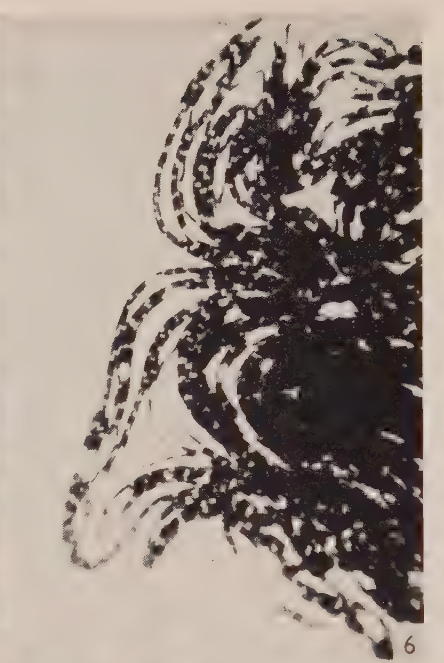
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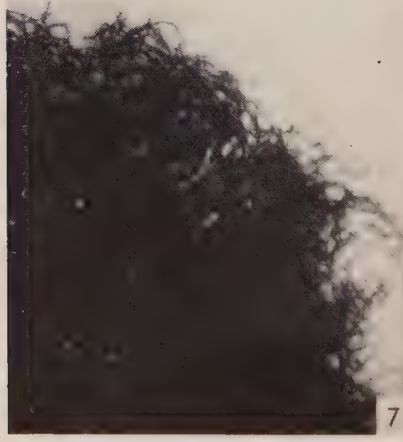
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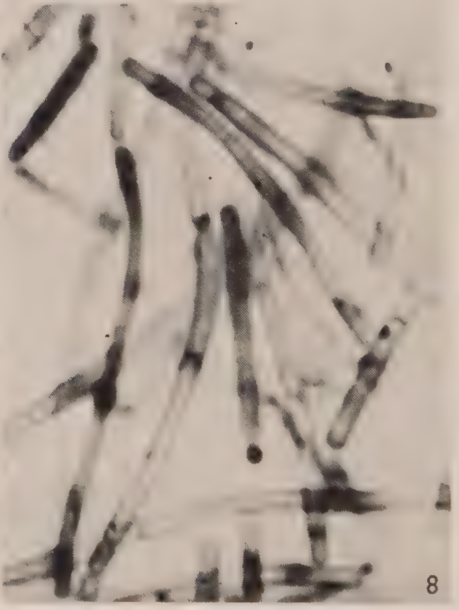
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G. H. G. Davis, K. A. Bisset and C. M. F. Hale—Characters of lactobacilli.
Plate 2

EXPLANATION OF PLATES

Figs. 1, 4, 6 and 7 are impression preparations of entire colonies; 12 hr. growth on medium 2; stained by dilute basic fuchsin; photographed at a magnification of × 500, except fig. 1, at × 200.

Figs. 2, 3, 5 and 8 are stained by Hale's method for cell walls; all at magnification × 3000.

PLATE 1

Figs. 1, 2. *Lactobacillus fermenti* type, representing the characteristic morphology of heterofermentative strains and also found in strains of *L. plantarum*. Short bacilli, 'smooth' colony.

Figs. 3, 4. Type of morphology observed in a minority of the heterofermentative strains, probably of the *Lactobacillus brevis* type and certain strains of *L. plantarum*. Markedly septate bacilli, longer than typical *L. fermenti* type; 'smooth' colony.

PLATE 2

Figs. 5, 6. *Lactobacillus casei-helveticus* type of morphology, also seen in some strains of *L. acidophilus*. The typical chain formation is not apparent in Fig. 5, but the curved bacilli are well shown, and the characteristic 'Medusa-head' colony clearly shows the bacilli in chains.

Figs. 7, 8. *Lactobacillus bulgaricus* and certain strains of *L. acidophilus*. Extreme filamentous cell-type, with few irregular septa. Tangled 'rough' colony.

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Inhibition of Histidine Uptake in *Neurospora crassa*

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SUMMARY: K12, K26 and K34 are three genetically distinct histidine-requiring mutants of *Neurospora crassa.* K12 and K26 are 0·69 units apart in the left arm of linkage group I, and K34 is in linkage group IV 32·6 units from 5531 (*pan*).

Response to histidine of these three mutants is markedly affected by the genetic background.

K26 accumulates two imidazole compounds, one of which may be histidinol and the other possibly compound II of Ames, Mitchell & Mitchell (1953). Like other histidine-requiring mutants K12, K26 and K34 are inhibited by certain other amino acids in combination with either arginine or lysine. The degree of inhibition is affected by the genetic background. The effect of the inhibitors is to prevent uptake of histidine from the culture medium. Histidine is accumulated from the culture medium against a concentration gradient and stored in the mycelium, from which it gradually disappears with growth. Inhibitors applied after accumulation have no effect on utilization of this stored histidine. Histidine is similarly accumulated by wild type and uptake is also prevented by a combination of the inhibitors arginine and methionine. The histidine requirement therefore serves to accentuate an effect which already exists in the wild type.

Amongst the considerable number and range of biochemical mutants originally obtained in *Neurospora crassa* (Beadle & Tatum, 1945), those needing histidine were conspicuously absent. Various causes may be surmised to explain this, such as impermeability of the cells to histidine or inhibition of such mutants by certain constituents of the medium. Assuming the last cause to be responsible, Lein, Mitchell & Houlahan (1948) succeeded in isolating a mutant which required histidine by screening on minimal medium supplemented with histidine alone. Haas, Mitchell, Ames & Mitchell (1952), who had obtained six additional histidine-requiring mutants, were able to assign these seven mutants to four different genetic loci. They showed also that the mutants could be distinguished by the imidazole derivatives they accumulated and that the inhibition observed on various complete media was due to certain combinations of amino acids.

The present report describes a further three mutants, genetically distinct from each other, their properties and the site of the amino acid inhibition. Evidence is presented to show that this inhibition is not a function of the histidine requirement or even consequent upon it, but that the histidine requirement merely makes apparent a situation which already exists in the wild type.

RESULTS

The mutants K12, K26 and K34 were obtained from Y8743-2-6A, a strain derived by Prof. M. Westergaard from Y8743 (Barratt & Garnjobst, 1949) and having the mutant genes peach-microconidial, colonial, a partial suppressor of colonial and albino-2. Microconidia of this strain were irradiated with ultraviolet light and the mutants recovered by the filtration enrichment technique (Catcheside, 1954).

Table 1 gives data for placing the three mutants in linkage groups, those in the first part being from random ascospores, scored for their ability to grow on minimal medium. Those able to grow (wild types) are one of the two expected classes of recombinant. The recombination percentage is calculated as twice the percentage frequency of these wild types. The second part of Table 1 gives data from ordered asci. K26 appears to be in the left arm of linkage group I, because no recombinants with the mating type locus appeared in 27 asci and each of the three second division segregations of K26 was also a second division segregation for mating type. K26 shows 24·5 % recombination with 15300 (*al*-2) and 0·32 % with mating type. This last figure is based on one recombinant in 309 random spores, so that the limits are wide, 0·0016–2·38 %.

Arginine 30300 (*arg*-3), probably in the left arm of linkage group I (Barratt, Newmeyer, Perkins & Garnjobst, 1954), is closely linked with K26, and of five wild type recombinants from the cross 30300*a* × K26A all were *a*. There are three possible orders for these three genes. (1) Mating type–30300–K26 is unlikely since a double cross-over would be necessary to give wild type recombinants which are *a*, and it is known that K26 is closely linked with mating type. (2) K26–mating type–30300 is also unlikely since one could expect some of the wild types to be *A*. (3) Mating type–K26–30300 is therefore the most probable order, only a single cross-over between K26 and 30300 being necessary to produce wild type recombinants which are *a*. K12 shows 0·69 % recombination with K26. From the cross K26 15300*A* × K12*a*, 24 tested recombinants, wild type with respect to histidine, were all *A*, six of them being albino and 18 wild type. The most probable order for K12, K26 and mating type is: mating type–K12–K26, based on the reasoning outlined above. The most likely place for 15300 is to the right of K26 since the majority of the wild type recombinants with respect to histidine are wild type with respect to 15300. K34 is in linkage group IV, 32·6 units distal to pantothenic 5531 (*pan*).

We have not assigned gene symbols because the genetic relationships of our mutants to those described by Haas *et al.* have not been determined. Three of their mutants, C94, C140 and T1710, are probably also in the left arm of linkage group I. A fourth, C141, is in linkage group IV, but it appears to be very close to pantothenic 34551 (Mitchell & Mitchell, 1954), which is considered to be allelic with 5531.

Growth Response

The original isolates were out-crossed to eliminate the undesirable colonial and microconidial genes and the new isolates differed considerably from each other in their response to histidine. This was measured as the dry weight attained after 4 days at 25° in 100 ml. Erlenmeyer flasks containing 20 ml. Fries 3 minimal medium supplemented with L-histidine. The mycelial mats were washed in distilled water, then dried at 80° for 5 hr. and weighed.

The reisolate of K26 gave half maximum growth on $1{\cdot}5\times10^{-4}$M-histidine, while that of K12 gave it on $0{\cdot}8\times10^{-4}$M-histidine and attained a far less total growth. K34 had a higher requirement for histidine, needing almost $3{\cdot}0\times10^{-4}$M-histidine for half maximum growth. The shapes of the response curves differ; the K34 stock in particular showed an *S*-shaped curve indicating a threshold requirement of histidine for effective growth. Such response curves, however, are very subject to the genetic background. The curves for K34 (Fig. 1) are from cultures of the four components of an ascus derived from a cross between two segregants of K34 which differed in total dry weight attained on an optimum amount of histidine. The curves for K12 (Fig. 2) represent the histidineless components of two asci from a cross K12 × wildtype. It is obvious from these data that the characteristics may be considerably altered by the genetic background, not only in the degree of response but in the nature of the response as shown by the shape of the curve.

Table 1. *Linkage data for K*12, *K*26 *and K*34

Cross	Linkage group of marker*	No. of wild type progeny	Total	Recombination (%)	Fiducial limits, *P*=0·05
K12 × K26	—	40	11,660	0·69	0·49–0·93
K12 × 66702 (*suc*)	I	58	2,472	4·69	3·59–6·03
K26 × 30300 (*arg*-3)	I	9	2,572	0·70	0·32–1·33
K26 × K91†	I	127	3,302	7·69	6·47–9·11
K34 × 5531 (*pan*)	IV	234	1,438	32·55	29·16–36·09

Cross	Ascus class (see below) 1	2	3	4	5	6	7	Total	Recombination (%)	Fiducial limits, *P*=0·05
K26 *A* × +*a*	24	—	—	—	3	—	—	27	0	0 – 5·25
K26 *al*-2 × ++(1)	17	2	19	2	1	—	1	42	24·48	19·08–29·88
K26 *al*-2 × ++(2)	32	—	21	—	1	—	—	54		
K34 × 5531	9	1	7	8‡	4	—	—	29	29·31	19·16–39·46

	Ascus class 1	2	3	4	5	6	7
a b × *a′b′*	*a b*	*a b′*	*a b*	*a b*	*a b*	*a b′*	*a′b′*
	a b	*a b′*	*a b′*	*a′b*	*a′b′*	*a′b*	*a b*
	a′b′	*a′b*	*a′b′*	*a′b′*	*a b*	*a b′*	*a b′*
	a′b′	*a′b*	*a′b*	*a b′*	*a′b′*	*a′b*	*a′b*

* See Barratt *et al.* (1954) for description of marker mutants and linkage groups.

† K91, one of our mutants, is probably allelic with 35203 (*ad*-3).

‡ Some of these may belong to class 7, since progeny from this cross were scored on germination appearance, which does not distinguish class 4 from class 7.

Accumulation

Accumulation of imidazole derivatives was determined chromatographically using the Pauly reaction essentially as modified by Ames & Mitchell (1952). Cultures were grown under the same conditions as were used for measuring response to histidine, but on a concentration of histidine giving half maximum growth, for 4 days or varying periods. Both the culture filtrate and the mycelium were tested. Extracts of the mycelium were made by boiling the washed mycelial mat from each flask for 1 min. in 2 ml. distilled water. Culture filtrates were evaporated to 1/10 of their volume *in vacuo* over sulphuric acid. Samples of the mycelial extracts and of the culture filtrates were spotted on no. 1 Whatman paper and run in a mixture of 3 parts of *n*-propanol to 1 part of 0·2 N-ammonia, or 3 parts of *n*-propanol to 1 part of N-acetic acid. After drying they were sprayed according to the method of Ames & Mitchell (1952), but with a slight modification in that the diazotized sulphanilic acid solution and the sodium carbonate solution were mixed in a proportion of 2:1 immediately before use and at 0°.

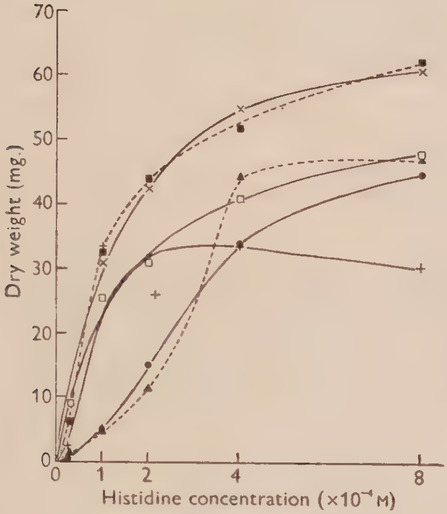

Fig. 1

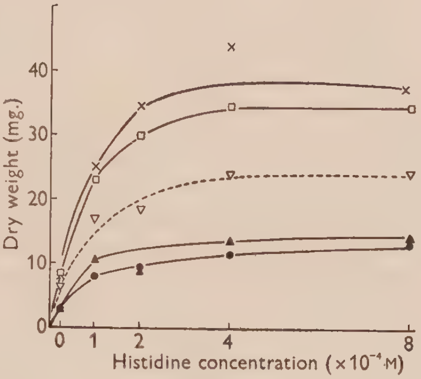

Fig. 2

Fig. 1. Yield curves of cultures derived from the four spores of a single ascus from a cross between two K 34 cultures. ■—■, ▲—▲, parent cultures. Yield measured as dry weight attained after growth for 4 days at 25° in 100 ml. Erlenmeyer flasks containing 20 ml. Fries 3 minimal medium supplemented with histidine.

Fig. 2. Yield curves of cultures from the histidine-requiring spores of two asci from the cross K 12 × wild type. ×—×, □—□, ascus 1; ▲—▲, ●—●, ascus 2; ▽—▽, K 12 parent.

Of the three mutants K 26 is the only one which shows any accumulation. There are characteristically two spots visible, the R_F's of which in the two solvents used are shown in Table 2. The beginning of accumulation coincides roughly with the time of disappearance of histidine from the mycelium. The double mutant K 26 K 34 shows no accumulation, suggesting that K 34 represents a block which occurs earlier in the synthesis than K 26. The double mutant K 12 K 34 likewise shows no accumulation but gives no information about the physiological relationships of the two genes concerned. The double mutant K 12 K 26 has not been obtained owing to the close linkage of these two genes. In respect of imidazole accumulation K 26 may correspond to

T1710 of Haas *et al.*, and K12 to their C140. Although K34 and C141 are in the same linkage group they disagree with respect to position and accumulation. There seems to be no *a priori* reason why the genetic background should not affect accumulation of shunt products either through causing a certain substance to accumulate or altering conditions so that there is no apparent accumulation. It must be admitted, though, that so far we have been unsuccessful in attempts to alter our histidine mutants with respect to their accumulations.

Table 2. *R_F values of histidine and imidazole compounds accumulated by* K26

3:1 propanol-0·2 N-NH_3	3:1 propanol-N-acetic acid	Substance
0·20	0·17	Histidine
0·57	0·24	Possibly histidinol
0·67	0·46	Possibly compound II (Ames *et al.* 1953)

Inhibition

Perhaps one of the most interesting features of mutants requiring histidine is their inhibition by complete medium. Haas *et al.* (1952) found that this was due to the presence of other amino acids, certain of which when combined with arginine, lysine, ornithine or α-amino-ϵ-guanidino caproic acid are completely inhibitory. Essentially the same situation exists for the mutants described here, although there were differences in effectiveness of various combinations. Inhibitors were added at a concentration of $3\cdot0 \times 10^{-4}$ M, while the histidine concentration was $1\cdot0 \times 10^{-4}$ M. The percentage inhibition was calculated as the percentage depression of the dry weight after 4 days growth at 25°. Data are shown (Table 3) for the effects of amino acids both singly and in combination with arginine and lysine on K12 and K26. L-Tryptophan, DL-methionine and L-tyrosine in combination with L-arginine or L-lysine were completely inhibitory, and glycine similarly combined was completely in-

Table 3. *Inhibition of* K12 *and* K26 *by various amino acids, singly and in combination with arginine or lysine, in the presence of histidine*

Inhibition expressed as percentage depression of the dry weight attained after 4 days growth at 25° in 20 ml. Fries 3 medium. Concentration of histidine $1\cdot0 \times 10^{-4}$ M, concentration of all other amino acids $3\cdot0 \times 10^{-4}$ M.

	Inhibitors alone		Inhibitors with L-arginine		Inhibitors with L-lysine	
Inhibitors	K12	K26	K12	K26	K12	K26
L-Arginine	35	65	—	—	—	—
DL-Methionine	17	78	100	100	89	100
L-Lysine	14	45	44	73	—	—
L-Tryptophan	24	36	100	100	100	100
Glycine	24	29	67	100	6	100
DL-β-Phenylalanine	20	26	25	73	17	39
L-Leucine	23	18	30	66	13	36
DL-Valine	19	11	27	58	17	58
DL-Isoleucine	9	9	23	21	12	29

hibitory for K26 but not for K12. In all instances of complete inhibition there was a positive interaction between the two amino acids in their inhibitory action. K34 behaved in essentially the same way as K12. K26 was more sensitive than the other two mutants since amino acid combinations were completely inhibitory when equal to the histidine concentration, whereas in K12 and K34 concentrations three times that of histidine were necessary.

These statements refer only to the original re-isolates of the respective mutants which have been used exclusively in the physiological studies described in this paper. However, the degree of inhibition may be altered by the genetic background, for example, K26 in a background largely of K12 obtained by two successive backcrosses behaves like K12 with respect to inhibition. While quantitative data are therefore of limited value, the relative effective concentrations of arginine and methionine for K26 are of some interest and are shown in Table 4. Arginine and methionine, both equal to or greater than the molar concentration of histidine, cause complete inhibition. Provided the methionine concentration is equal to the histidine, inhibition is still complete even though the arginine concentration is as low as one-eighth that of the histidine. But if the concentrations are reversed so that the arginine concentration is greater than that of histidine while the methionine concentration is reduced to one-eighth, the inhibition observed is only equal to that with arginine alone.

Table 4. *Inhibition of* K26 *by various concentrations of arginine and methionine in the presence of* $1{\cdot}0 \times 10^{-4}$ M-*histidine*

Inhibition expressed as percentage depression of the dry weight attained after 4 days growth at 25° in 20 ml. Fries 3 medium.

DL-Methionine concentration 10^{-4} M ×	L-Arginine concentration								
	0	0·125	0·25	0·5	1·0	2·0	4·0	8·0	16·0
0	Not tested				24	61	66	71	76
0·125					17	63	67	73	71
0·25					30	63	69	75	73
0·5					48	71	73	70	67
1·0	15	40	40	40	Complete inhibition				
2·0	64	Complete inhibition							
4·0	86								
8·0	94								
16·0	95								

For the combinations arginine + methionine and arginine + tryptophan the inhibition is competitive. Other inhibitory combinations were not tested. There is some evidence that the various amino acids supplement each other in inhibition. In the presence of $1{\cdot}0 \times 10^{-4}$ M-histidine, a mixture of three inhibitors, $0{\cdot}5 \times 10^{-4}$ M arginine + $1{\cdot}0 \times 10^{-4}$ M methionine + $1{\cdot}0 \times 10^{-4}$ M tryptophan is completely inhibitory to K12 and K34, while the pairs arginine + methionine or arginine + tryptophan in the same concentrations as above are non-inhibitory. All three combinations are inhibitory to K26.

SITE OF INHIBITION

The question naturally arises whether this inhibition is concerned with uptake of histidine by the cell or with utilization of the histidine at some site within the cell. A semiquantitative estimation of the distribution of histidine between culture filtrates and mycelial extracts can be made by chromatography. It appeared from the chromatograms that the mutants accumulate histidine from the medium against a concentration gradient. This histidine is then extractable from the mycelium during the growing period in gradually diminishing amounts until it has completely disappeared. Superficially the histidine appears to be accumulated and stored in the mycelium to be used as required. Whether or not it is stored as histidine is not known, but at least it can be extracted again from the cells and behaves as histidine on a chromatogram. Arginine and methionine were also estimated chromatographically being localized by ninhydrin, and they appear to behave in a similar fashion to histidine. Such accumulation and storage is not a function of the mutants only but is also a property of the wild type.

Since histidine is accumulated it was argued that if the inhibitory action is on uptake and not utilization, inhibitors added after histidine had been taken up by the mycelium should be ineffective in preventing growth. An experiment to test this was designed using K26, with arginine and methionine as inhibitors. The data are presented graphically in Fig. 3. Three main conclusions are demonstrated: (1) the addition of inhibitors after 2 days growth in the presence of histidine has no effect on the growth attained on this prior supply of histidine; (2) when arginine and methionine are present in concentrations below those required for inhibition growth is stimulated; and (3) inhibitors added after initial growth on histidine prevent the utilization of histidine added together with them, and this inhibition persists for 5 days from the addition of the inhibitors and extra histidine. These results suggest that the inhibitors prevent histidine from entering the cell rather than prevent it being utilized once there.

Similar experiments with K12 and K34 showed only a very slight effect of the inhibitors, growth of treated cultures rapidly equalling the controls. Both K12 and K34 had previously been shown to be less sensitive to inhibitors than K26, and measurement of dry weight would be unable to detect any inhibition which did not last at least 2 days. Consequently, a more sensitive measure of histidine uptake was used. Essentially the method consisted in treating the culture filtrates and mycelial extracts with diazotized sulphanilic acid and sodium carbonate (Haas *et al.* 1952) and measuring the density directly on a colorimeter. Cultures were grown in 20 ml. Fries 3 medium containing $1{\cdot}0 \times 10^{-4}$ M-histidine for 36 hr., by which time all the histidine had been removed from the medium. The mycelial pads were gently squeezed against the side of the flask and then transferred to fresh medium containing either $3{\cdot}0 \times 10^{-4}$ M-histidine alone or this same concentration of histidine with arginine and methionine each in concentrations of $9{\cdot}0 \times 10^{-4}$ M. Two flasks were taken at each sampling. From one the mycelium was removed, washed and dried at

80° for 5 hr. for dry-weight measurement. From the other the mycelium was washed, squeezed dry, suspended in 2 ml. water and boiled for 1 min. A sample was chromatographed as described above. Another sample was diluted 100 times and a 3 ml. sample was mixed with diazo reagent. The density was measured 1 min. after the time of mixing, using an EEL photo-electric colorimeter with filter 623. Culture filtrates were treated similarly after diluting 10 times. Samples of culture filtrates were concentrated 10 times and chromatographed as described above.

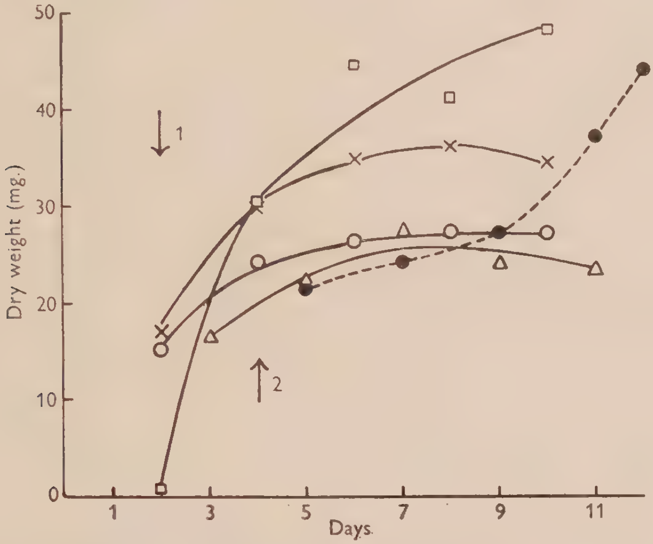

Fig. 3. Effect of arginine and methionine on the dry weight of K26. The dry weight was measured after growth at 25° in 100 ml. Erlenmeyer flasks containing 20 ml. Fries 3 minimal medium with the following additions: *(a)* ○—○, $1 \cdot 0 \times 10^{-4}$ M-histidine; *(b)* ×—×, $2 \cdot 0 \times 10^{-4}$ M-histidine; *(c)* △—△, $1 \cdot 0 \times 10^{-4}$ M-histidine, arginine and methionine (each $1 \cdot 0 \times 10^{-4}$ M) added after 2 days; *(d)* ●—● as for *(c)* and $1 \cdot 0 \times 10^{-4}$ M-histidine added after 4 days; *(e)* □—□ $2 \cdot 0 \times 10^{-4}$ M-histidine + arginine and methionine (each $1 \cdot 0 \times 10^{-4}$ M); ↓1, arginine and methionine added; ↑2, extra histidine added.

Certain constituents of the culture medium react with the diazo reagent to form yellow compounds which can be detected on chromatographs. These will contribute to the recorded density as shown by the culture medium blank. They will doubtless change during the course of the experiment so that the density measured will not be due entirely to histidine, although it will contribute the greater part. The mycelial extracts do not suffer from this disadvantage since none of these yellow coloured compounds can be detected chromatographically in them.

In K12 (Fig. 4) histidine disappears from the culture medium after 40 hr. in the absence of inhibitors, but in the presence of inhibitors does not commence to disappear until 60 hr. and disappearance is not complete until some time after 70 hr. In K34 (Fig. 4) the inhibition is overcome more rapidly, uptake without inhibitors being complete at 30 hr. and with inhibitors at 50 hr.

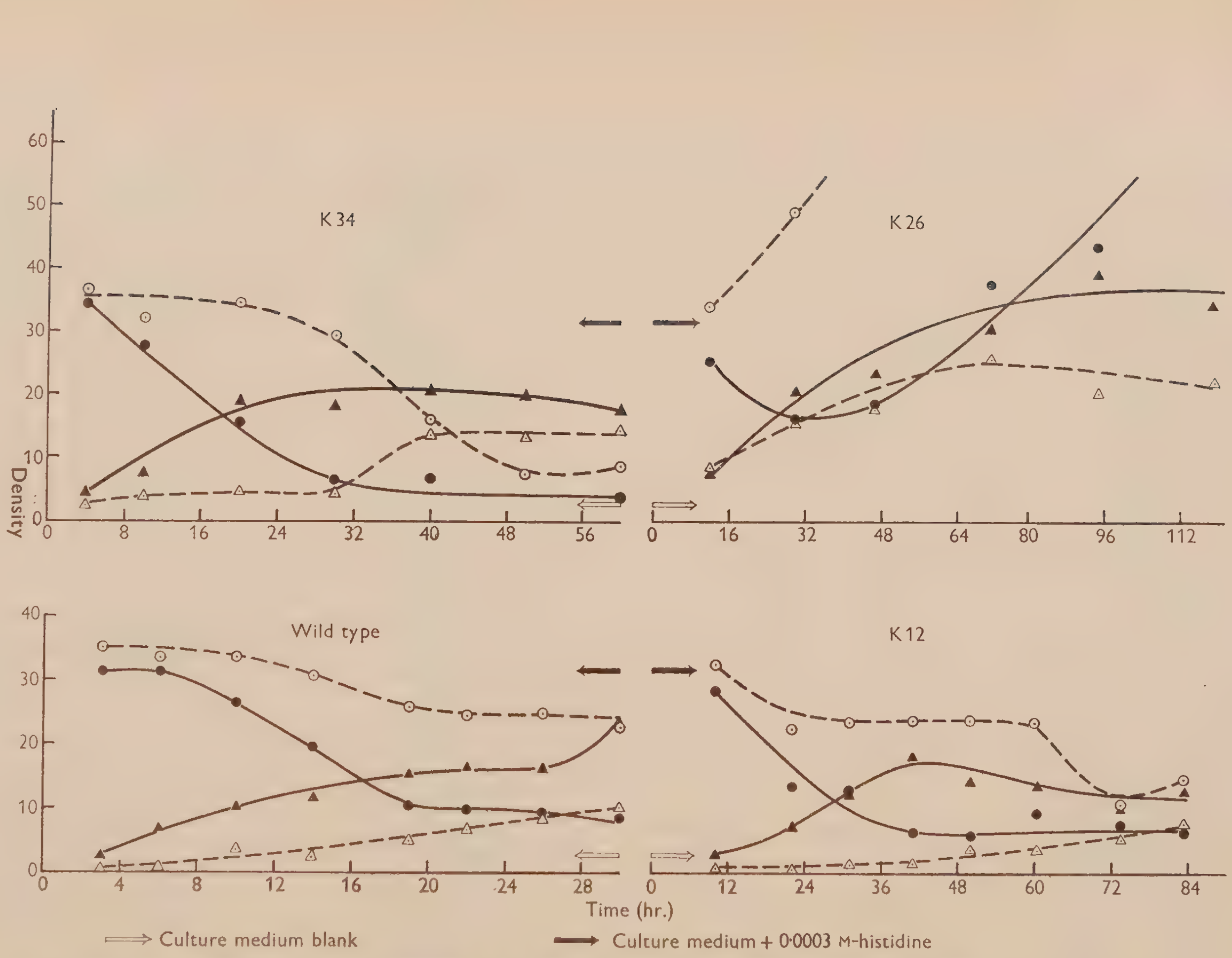

Fig. 4. Effect of inhibitors on histidine uptake in K 12, K 26, K 34 and wild type. Histidine concentration measured on an EEL colorimeter, filter 624, as the density of samples from culture filtrates and mycelial extracts after treatment with diazotized sulphanilic acid, 1 min. from the time of mixing. Initial histidine concentration in culture medium $3{\cdot}0 \times 10^{-4}$ M. ●—●, culture filtrates without inhibitors; ⊙- - - ⊙, culture filtrates with inhibitors (arginine and methionine, each $9{\cdot}0 \times 10^{-4}$ M); ▲—▲, mycelial extracts without inhibitors; △ - - - △, mycelial extracts with inhibitors (arginine and methionine, each $9{\cdot}0 \times 10^{-4}$ M).

In a similar experiment with K26 (Fig. 4) changes in histidine concentration as determined on the colorimeter are obscured by the accumulation of imidazole compounds, but separation of the different substances on the chromatogram shows that changes in histidine concentration follow the same pattern as in K12 and K34.

The question then arises whether inhibition of uptake is a direct consequence of the histidine requirement or whether the uptake of histidine by the wild type is similarly affected by inhibitors. Fig. 4 and Pl. 1 show the results of a similar experiment on wild type. Obviously the inhibitors do prevent uptake of histidine by wild type, but in the absence of a histidine requirement growth is not impeded, the inhibitors are rapidly removed from the medium and their effect is only transitory.

It is possible to make a rough estimate of the relative concentrations of recoverable histidine in the culture medium and in the mycelium at equilibrium from the graphs for K12, K26, K34 and wild type. The concentration in the mycelium is of the order of two to three times that in the culture fluid. This will be an underestimate since histidine is being utilized for growth.

Similar accumulation of histidine occurs in cells of rat intestine. Agar, Hird & Sidhu (1954) found that the ratio between the histidine content of the external fluid and the intestine was 2·96 when the external concentration of histidine was 5 μmole/ml.; the ratio decreased with increasing concentration of histidine. D-Histidine was not accumulated. They also found that cyanide and 2·4-dinitrophenol inhibited the uptake. Wiseman (1953) showed that L-alanine, L-phenylalanine, L-methionine, L-histidine and L-isoleucine were actively transported against a concentration gradient in rat intestine, while glutamic and aspartic acids were transported by simple diffusion. The D isomers were not actively transported.

The phenomenon of accumulation of amino acids by cells may be of widespread occurrence, but differences in behaviour with respect to individual amino acids seem to occur between different organisms. In *Streptococcus faecalis* Gale (1947) found that histidine was not accumulated but that a source of energy was necessary for uptake to occur. Lysine, on the other hand, which appeared to enter the cell by simple diffusion was actively accumulated, and he suggests that it was held in the cell by electrostatic attraction. Najjar & Gale (1954) have found that the presence of basic or acidic amino acids in the medium reduces the uptake of lysine by the cells, and likewise presence of these amino acids inside the cell reduces the amount of lysine which will migrate out of the cell. This appears to be somewhat similar to the amino acid inhibitions of *Neurospora crassa*. In *Streptococcus faecalis* the acidic and the basic amino acids seem to be equally effective, but combinations of basic + neutral amino acids which are most effective in *Neurospora crassa* were not tried.

The nature of the amino acid inhibitions in *Neurospora crassa* is not known. Perhaps a likely place for such competition is the cell membrane, and attempts were made to alter the nature of this membrane by treatment with various detergents, but without positive effects on the inhibition.

Two of the essential constituents of the inhibitor complex are concerned in mutual inhibitions in other mutant strains of *Neurospora crassa*. Certain lysineless mutants are competitively inhibited by exogenous arginine (Doermann, 1944), while some strains requiring arginine are inhibited by exogenous lysine (Houlahan & Mitchell, 1948; Srb, 1953). No experiments are reported which would distinguish between inhibition of uptake and of utilization in these strains.

Pontecorvo (1952), however, has found similar arginine-lysine inhibitions in Aspergillus and suggests that the site of inhibition is within the cell but outside the nucleus. This suggestion is based on the findings that diploids involving the two mutants are not affected by exogenous inhibitors, whereas heterocaryons between them, while having no requirement, are nevertheless sensitive to inhibitors. No doubt the heterocaryon is subject to dissociation through change in the medium, whereas the diploid is necessarily more stable. Consequently, this evidence is not critical for showing the site of inhibition.

We are indebted to Professor A. E. Alexander for suggesting the trial of detergents and for the supply of sodium hexadecyl sulphate, cetyl pyridinium chloride, Aerosol M.A., and ethylene oxide/cetyl alcohol (30:1), all of which were ineffective in modifying the inhibitions.

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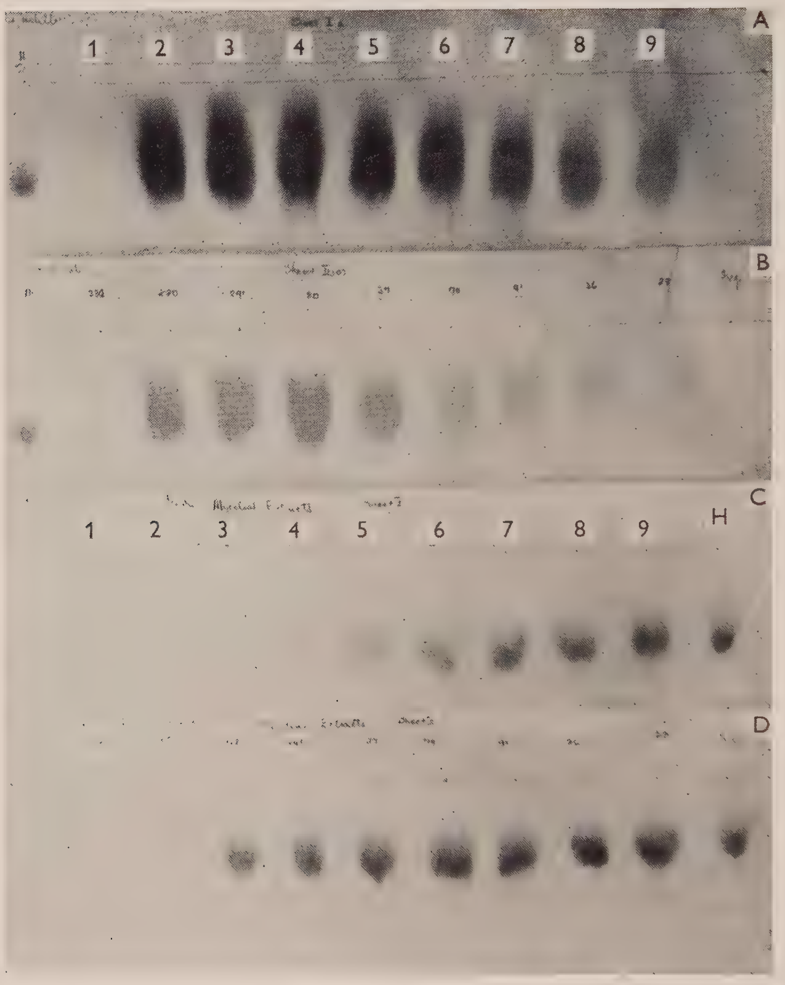
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M. J. Mathieson and D. G. Catcheside—Inhibition of histidine uptake.
Plate 1

(*Facing p.* 83)

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EXPLANATION OF PLATE

Chromatograms showing histidine content of culture filtrates (A and B) and of mycelial extracts (C and D) during growth of wild type mycelium in a minimal medium supplemented with histidine alone (B and D) or with histidine plus the inhibitors arginine and methionine (A and C). Initial histidine concentration in the culture medium $= 3{\cdot}0 \times 10^{-4}$M. Initial concentration of each inhibitor in the culture medium $= 9{\cdot}0 \times 10^{-4}$M. H = histidine control. 1 = culture medium blank in A and B, and extract of mycelium at start of the experiment in C and D. 2–9 = samples taken at 3, 6, 10, 14, 19, 22, 26 and 30 hr. from start of the experiment.

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An Electron Microscope Examination of the Initial Cell Stage in *Streptomyces* spp.

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SUMMARY: Electron microscope observations were made on submerged cultures in defined medium of two species of the genus *Streptomyces*. The evidence obtained confirms the supposition of Klieneberger-Nobel (1947), with surface cultures of four other species, that at an early stage in the life history of the organism concerned, fusion occurs between portions of the same or different hyphae, culminating in the formation of 'initial cells'. In *S. griseus* initial cells were observed at 26 hr. and as late as 6 days. Certain unidentified bodies were observed. These may be related to initial cell formation.

It has been known for some time that species of the genus *Streptomyces* show two different growth phases—the substratum mycelium and the aerial mycelium (Ørskov, 1923). These phases were renamed the primary and the secondary mycelium, respectively, by Kleineberger-Nobel (1947); her terminology will be used in this paper. The recognition of diphasic growth, along with a persistent branching filamentous habit and reproduction from spores, suggests, according to Bisset & Moore (1949), a clear separation from the other Actinomycetales (*Bergey's Manual*), and on this basis the 'four sporing Actinomycetes' described by Klieneberger-Nobel in her paper may be considered as species of the genus *Streptomyces*. This author made the first critical examination of the life cycle of these organisms as it occurs in surface cultures on a solid agar medium or on a liquid medium. By combining a staining method based upon that of Robinow (1942) with careful microscopical examination, she found a marked difference in the two phases of growth in the species examined. The primary mycelium, developed from germinating spores, is septate and profusely branching, and the individual hyphae, which are somewhat less than 1 μ. in diameter, contain 'granular or rod-shaped chromatin bodies'. The secondary spore-bearing mycelium, as she describes it, arises directly from the primary mycelium in agglomerations of the latter and from points of fusion which Klieneberger-Nobel terms 'initial cells'; it is distinguished in that it is thicker, branches less profusely, eventually breaks into single cells or spores and has a more clearly defined internal structure. According to Klieneberger-Nobel the spores are developed after fusion and organization of the cellular material, processes whose exact significance is not yet apparent. Klieneberger-Nobel suggested that the complete cycle might represent alternating generations of haploid primary mycelium with diploid secondary mycelium, with sexual fusion occurring in the initial cell, but stated that 'in order to prove this an analysis of "chromosome" content complemented by observations proving the actual fusion of chromatin would be required. Such evidence will be difficult to obtain, since all the elements concerned are of exceedingly small dimensions'.

Similar observations were made on the closely related genus *Micromonospora* by Morris (1952); and more recently McGregor (1954), in describing certain aspects of the life cycle of an unspecified *Streptomyces* strain, generally agreed with Klieneberger-Nobel's findings, but differed in that he considered that initial cells are not formed from 'nests of filaments' and appeared to arise, not from points of hyphal fusion, but from single swellings in the hyphae or as short appendages. Klieneberger-Nobel's conclusions were strongly disputed by Erikson (1949), on the grounds that hyphal agglomerations are artefacts inevitably caused in the preparation of the microscopical specimens, further, she stated that in her experience, 'in no circumstance has any fusion been detected between filaments of the same or different colonies'. The secondary mycelium, in Erikson's view, arises directly from the primary mycelium, without the intervention of an initial cell stage. However, Erikson did mention, without comment, that Carvajal (1946) observed hyphal fusion during electron microscope studies of *Streptomyces griseus*, though this author provided no photographic or descriptive evidence for this statement. It is clear that much of the doubt as to the nature and function of the 'initial cell' lies in the difficulty of interpreting the appearance of objects close in size to the limit of resolution of an ordinary microscope. The purpose of the present paper, which describes electron microscopic observations on streptomycin- and vitamin B_{12}-producing strains of *S. griseus* and of a strain of *S. aureofaciens*, is to present alternative evidence for the existence of these structures. In addition, we have recorded certain other observations on the morphology of *Streptomyces* spp.

METHODS

Most of our observations were made on material grown in agitated submerged culture in which, with certain media (Carvajal, 1947), sporulation readily occurs. The cycle of events leading to sporulation is then fundamentally the same as that observed for agar surface cultures.

Cultures were grown in shaken 250 ml. conical flasks containing 40 ml. medium, of which two kinds were used. The first (urea medium) consisted of glucose, 5 g.; brown sugar, 10 g.; urea, 2 g.; KH_2PO_4, 2 g.; with water to 1000 ml. and had a pH value of 6·8 (after sterilization at 120° for 20 min.). The second (AS medium) consisted of glucose, 25 g.; $MgSO_4.7H_2O$, 1 g.; $(NH_4)_2SO_4$, 4 g.; $NaNO_3$, 5 g.; NaCl, 5 g.; KH_2PO_4, 0·5 g.; K_2HPO_4, 1 g.; $CaCO_3$, 0·45 g.; trace elements in the form of soluble salts of iron, manganese, zinc, copper and molybdenum at the rate of 3 p.p.m. of each element; with water to 1000 ml. This had a pH value of 7 (after sterilization at 120° for 20 min.). Flasks were inoculated with spores from surface cultures of the organism concerned and grown at 28° on a rotary shaker (200 r.p.m.).

Electron microscope examinations of *Streptomyces griseus* and *S. aureofaciens* were made exclusively on formalin-fixed material. Material for visual observations was fixed in osmium tetroxide vapour and stained by the HCl Giemsa method of Robinow (1942). Random electron microscope observations were made directly, without metal shadow-casting, on samples of *S. griseus* ranging from 26 hr. to 6 days in age, and of *S. aureofaciens* 46 and 53 hr. old. All samples were mounted, air-dried, on formvar films after washing six times by centrifugation in distilled water, a procedure found to be necessary to eliminate traces of dissolved medium constituents which interfered with the clarity of the electron micrograph. Most of the observations were made at an accelerating voltage of 60 kV., though some were made at 80 kV. The resulting prints vary in magnification between 2500 diameters and 12,000 diameters, but that recorded is to some extent dependent on the accelerating voltage, which, though stable over an operating period, may vary slightly from day to day, so that the figures are not exact, but near enough for comparative purposes. The ordinary light photomicrographs are reproduced at a magnification of 2000 diameters. The electron microscope used was the Philips instrument in the Department of Biophysics at Leeds University, and the ordinary photomicrographs were made with a Zeiss 1·3 N.A., ×100 fluorite oil-immersion lens, a Leitz ×10 Periplan eyepiece, a 1·4 N.A. Zeiss aplanatic condenser and a Leitz Makam camera attachment, with Köhler illumination and green or blue Ilford filters.

RESULTS

With visual light examination, the appearance of the initial cell stage is illustrated in Pl. 1, fig. 1, which is of *Streptomyces griseus* primary mycelium aged 48 hr. Four main features are observable, and are indicated on the photomicrograph by the lettered annotations: (*a*) initial cells may arise at the point of fusion of separate hyphae; (*b*) they may arise in an intercalary position on individual hyphae without fusion with other hyphae; (*c*) they develop as short appendages to individual hyphae; (*d*) they may break away free from the mycelium. In whatever position they arise, initial cells appear to fulfil the same function of initiating the secondary mycelium, as illustrated in Pl. 1, fig. 2, where the first stage in 'germination' of an initial cell is clearly seen. With a gradual elongation and thickening of the 'germ-tube' the hypha then assumes the characteristic appearance of secondary mycelium (Pl. 1, fig. 3; see also Klieneberger-Nobel, 1947, pl. 2, fig. 13). In the species described by Klieneberger-Nobel, hyphal fusion leading to initial cell formation seems to occur in a tangled 'nest of filaments', but we observed this feature only occasionally (Pl. 1, fig. 4). The functional significance of this is not obvious; it may be a fortuitous agglomeration of free initial cells.

When viewed directly with an electron microscope after formalin-fixation, the mycelium varies in its opacity to the electron beam; internal structure is only occasionally visible, but useful information may be obtained from 'shadow' impressions. Generally the internal structure revealed has the appearance shown in Pl. 2, figs. 7, 8. Material dense to transmitted light is not necessarily dense to electrons, but the texture here is reminiscent of vacuolated cytoplasm as observed by conventional methods. The number and diversity in size of the less dense patches suggest that they are vacuoles and preclude their being nuclei. The various features observed will be dealt with under subheadings; as they have commonly been observed in specimens of either species, this will not be specified in the text, but may be found from the legends of the figures.

Hyphal fusion. Evidence for the occurrence of hyphal fusion is strengthened, as will be seen by reference to the portions indicated by arrows in Pl. 1, fig. 5, and Pl. 2, fig. 6. It might be argued that the hyphae simply assumed close parallel positions in the preparation of the specimen, and this undoubtedly does occur through surface tension effects during drying on the electron microscope mount. However, in direct observation on the screen it was possible to differentiate between this appearance and that of Pl. 1, fig. 5, and Pl. 2, fig. 6, where the bridge between the hyphae consists of a short outgrowth from one lying close to the other, presumably just before fusion takes place along the contact boundary. Reference to Pl. 1, fig. 5, will show that there is an unmistakable thickening in the adjacent (arrowed) portion, which may indicate a positive reaction caused in one hypha by the proximity of another.

Initial cells. It seems that hyphal fusions between different hyphae, as described above, or between different portions of the same hypha, in which the eventual coalescence of material has varying degrees of complexity, are often the first stages in the formation of initial cells: Pl. 2, figs. 7–9, and Pl. 3, fig. 10, illustrate this thesis. The bodies illustrated represent the first stages of initial cell formation from double (Pl. 2, fig. 7), treble (Pl. 2, fig. 8) and multiple (Pl. 2, fig. 9) hyphal contacts, and Pl. 3, fig. 10 represents a late stage of an initial cell limited to one hypha (see also Pl. 1, fig. 1*b*).

Pl. 2, figs. 7, 8, confirm the opinion formed from visual light observations (Pl. 1, fig. 1*d*, and McGregor, 1954) that initial cells may become detached from the main mass of mycelium. This seems to take place at thinned portions of the hyphae, which occur at irregular intervals along their length (Pl. 2, fig. 9; Pl. 3, figs. 11, 12), thus accounting for the tapered extremities in Pl. 2, figs. 7, 8.

In our cultures the presence of initial cells is not limited to the early stages of the fermentation, although it is limited to the early stages of the life cycle. This is borne out by their presence as late as 6 days (Pl. 2, figs. 7, 8) after inoculation of cultures of *Streptomyces griseus*, and indeed this is not surprising, since Carvajal (1947) in his early study of submerged cultures found that spores formed under such conditions can germinate late in the same fermentation and regenerate fresh mycelium. Clearly, in our material it is this fresh primary mycelium that has given rise to the late-formed initial cells.

Unidentified objects. Several bodies seen could not immediately be related to any views based upon visual light examinations. One of these is shown in Pl. 3, fig. 13 (arrowed): it is ovoid in shape, transparent to electrons and flanked by two thickened portions of the parent hypha. From each end arises a single short filament of the same thickness as the unthickened parent hypha, forming a crossed pair at one side of the ovoid. It may be that the whole apparatus represents the germination of a single initial cell *in situ* in its parent hypha, but the direction in which the two short filaments (possibly germ tubes) are growing is hardly what one would expect if this were so. An alternative possibility is that the structure may represent hyphal regeneration from a damaged portion.

In other examples (Pl. 3, figs. 11, 12) portions of hyphae opaque to electrons give way to sections that are electron transparent and of unknown significance.

DISCUSSION

The presence of a fusion initial cell stage in the life history of *Streptomyces* spp. prompts speculation as to its function, for it is suggestive of a haploid primary mycelial stage leading after nuclear fusion in the initial cell to a diploid secondary mycelial stage. Since it seems clear from the work described above that initial cells can arise either within a single hypha or from fused portions of two or more hyphae, then nuclear fusion, if it takes place, is likely to be an intra-mycelial as well as an inter-mycelial event.

An hypothesis of a haploid/diploid alternation was tentatively put forward by Klieneberger-Nobel (1947), who qualified it with the reservation, previously quoted, that the evidence would be difficult to obtain because of the exceedingly small size of the elements concerned. We agree with this view, and indeed extend it, for it is thought that visual microscope methods are unlikely to resolve the problem with certainty. Nor would electron microscope observation; by the nature of preparation by desiccation and the disruptive effect of the electron stream on cellular material, cytological observations by these methods are difficult to interpret. In view of this doubt, it would be preferable to investigate the problem by genetical rather than cytological methods. By such means, based upon the methods of Pontecorvo (1953) for the genetical analysis of homothallic fungi, it might be possible to establish the presence or otherwise of a true sexual fusion.

If there were a true alternation of generations, then at some point before spore formation, or soon after, there would be a restoration of the haploid state through the agency of a meiotic division. Indeed, there is some evidence that meiosis may not be a pre-sporulation event, but that it occurs at least after spore maturation. This is suggested by experimental work carried out by us on spore material from surface cultures of *Streptomyces griseus* in which attempts were made to produce nutritional mutant strains by the usual techniques (Pontecorvo, 1953). No nutritional mutants were retrieved from among 1600 ultraviolet irradiation survivors. Their complete absence could be ascribed to a variety of causes, of which the most likely are a high resistance to the mutagenic effects of ultraviolet light or spores that are diploid or multinucleate.

The first of these possibilities is thought to be unlikely, for here and elsewhere the mutagenic effect of ultraviolet light on *Streptomyces griseus* has been demonstrated for mutations involving a change in streptomycin or vitamin B_{12} productivity or pigment formation or morphological changes.

On the other hand, if the spores irradiated were diploid or multinucleate the possibility of retrieving nutritional mutants by the methods we employed would be small, since in a diploid cell, two mutations involving each allele of a particular locus would be necessary for the phenotypic expression of a particular growth factor requirement, whereas in a haploid binucleate cell, for example, two mutations at the same locus in each nucleus would be required. There is, however, cytological evidence that the spores of *Streptomyces* spp. are not multinucleate (Klieneberger-Nobel, 1947; McGregor, 1954; our observations).

We wish to express our thanks to Professor R. D. Preston of the Department of Biophysics, Leeds University, for the facilities he provided; to Mr G. W. Ripley and Mr R. W. McWilliam for technical assistance, and to Dr B. Northam and Dr A. Rhodes for the supply of material.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. *S. griseus*: primary mycelium at 48 hr. from submerged culture showing initial cells at *a*, *b*, *c*, *d*, and other positions. Osmium tetroxide fixation (HCl-Giesma staining). Visual light, ×2000. Urea medium.

Fig. 2. *S. griseus*: primary mycelium at 40 hr. from submerged culture, showing germinating initial cell. Osmium tetroxide fixation (HCl-Giemsa staining). Visual light, ×2000. A.S. medium.

Fig. 3. *S. griseus*: primary mycelium at 44 hr. from submerged culture showing elongation of secondary mycelium from an initial cell. Osmium-tetroxide fixation (HCl-Giemsa staining). Visual light, ×2000. A.S. medium.

Fig. 4. *S. griseus*: primary mycelium at 40 hr. from submerged culture, showing agglomeration of hyphae. Osmium tetroxide fixation (HCl-Giemsa staining). Visual light, ×2000. Urea medium.

Fig. 5. *S. aureofaciens*: primary mycelium at 53 hr. from submerged culture, showing hyphal fusions. Electron micrograph, ×6000. Urea medium.

PLATE 2

Fig. 6. *S. griseus*: primary mycelium at 53 hr. from submerged culture, showing hyphal fusions. Electron micrograph, ×5000. Urea medium.

Fig. 7. *S. griseus*: first stage of initial cell formation from double hyphal contact. From 6-day submerged culture. Electron micrograph, ×10,000. (Inset: diagram illustrating conformation of hyphae.) Urea medium.

Fig. 8. *S. griseus*: first stage of initial cell formation from treble hyphal contact. From 6-day submerged culture. Electron micrograph, ×12,000. (Inset: diagram illustrating conformation of hyphae.) Urea medium.

Fig. 9. *S. griseus*: early stage initial cell formation from multiple hyphal contacts, showing thinning portions of hyphae. From 26 hr. submerged culture. Electron micrograph, ×5000. A.S. medium.

PLATE 3

Fig. 10. *S. aureofaciens*: late stage initial cell formation within a single hypha. From 53 hr. submerged culture. Electron micrograph, ×10,000. Urea medium.

Fig. 11. *S. aureofaciens*: thinned out portions of hyphae and electron transparent portions. From 46 hr. submerged culture. Electron micrograph, ×5000. Urea medium.

Fig. 12. *S. aureofaciens*: thin portions of hyphae with electron transparent sections. From 26 hr. submerged culture. Electron micrograph, ×2500. Urea medium.

Fig. 13. *S. aureofaciens*: electron transparent 'unidentified body'. From 53 hr. submerged culture. Electron micrograph, ×6000. Urea medium.

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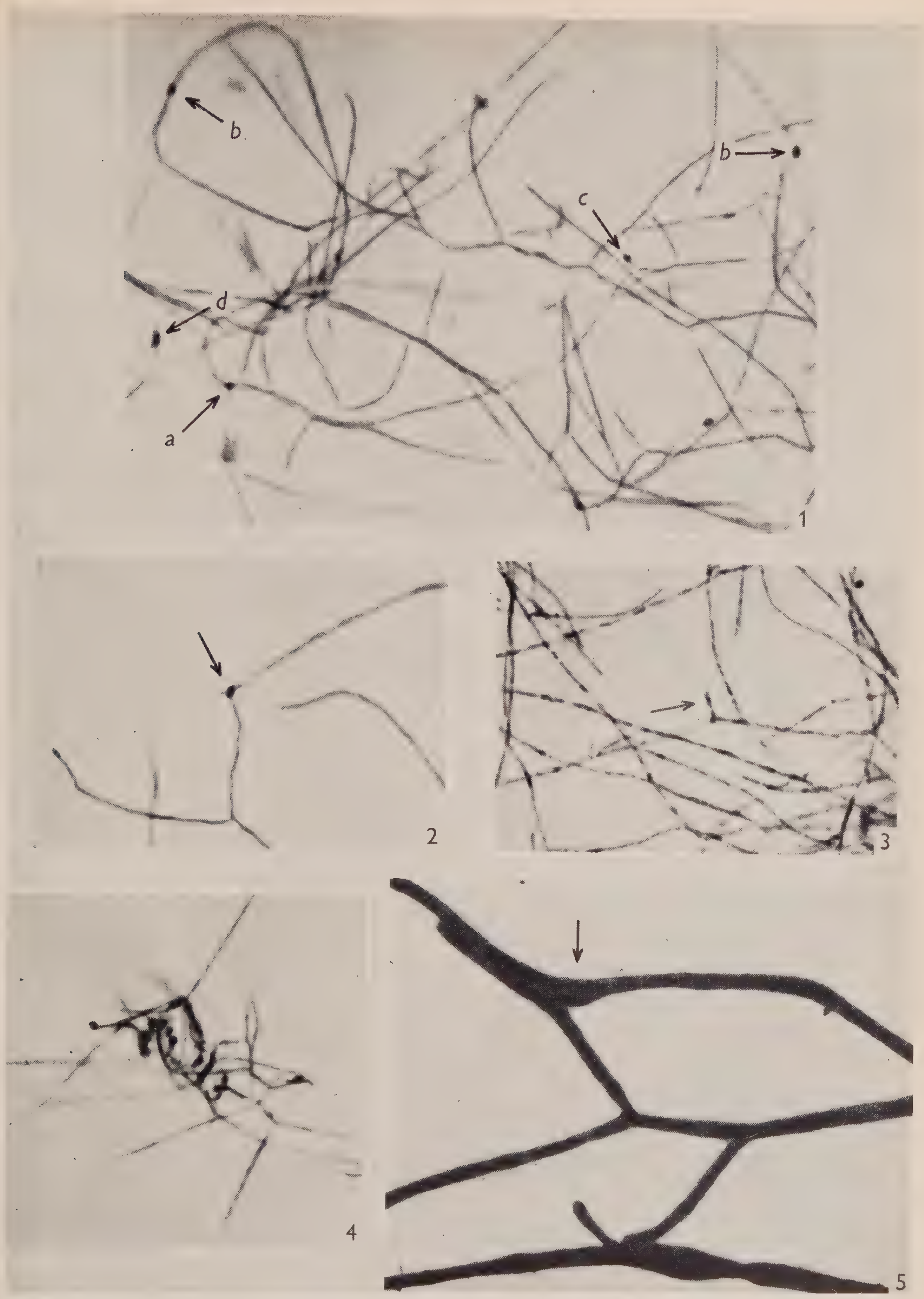

P. B. Dickenson and K. D. Macdonald—Initial cells in *Streptomyces* spp. Plate 1

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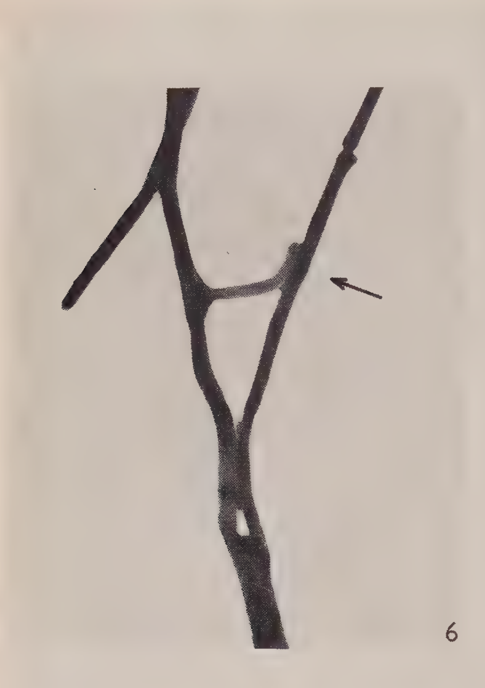

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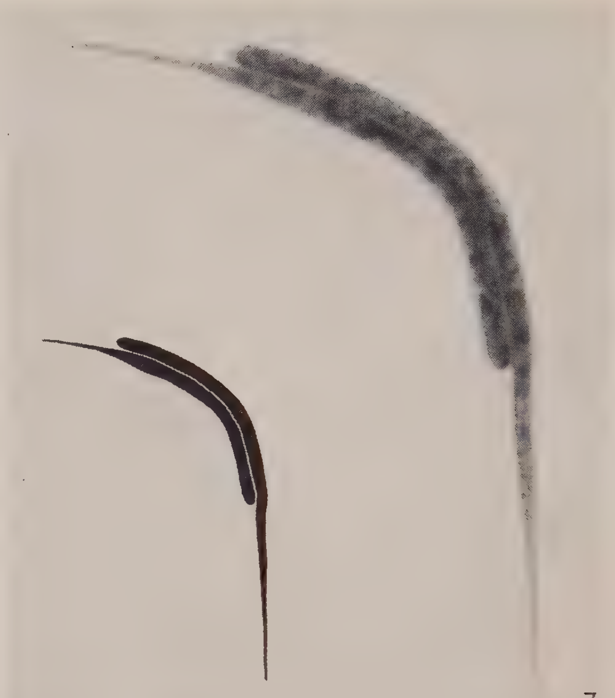

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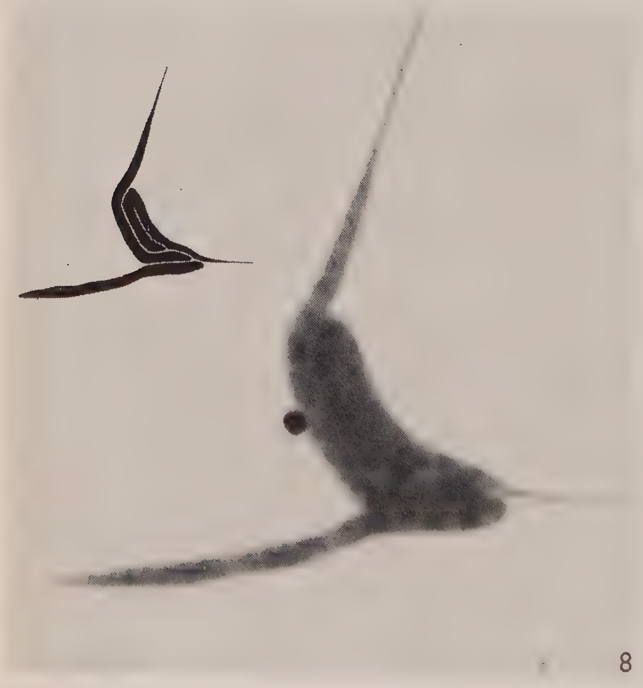

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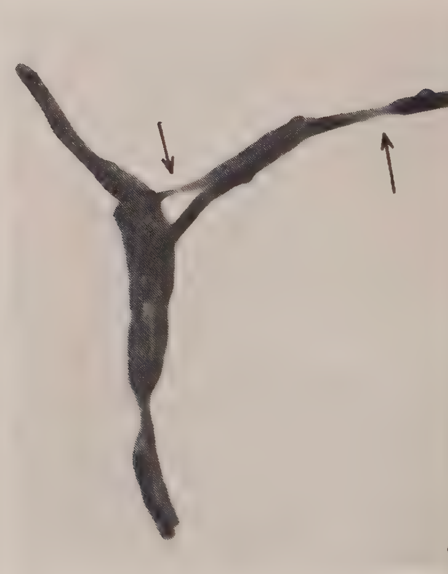

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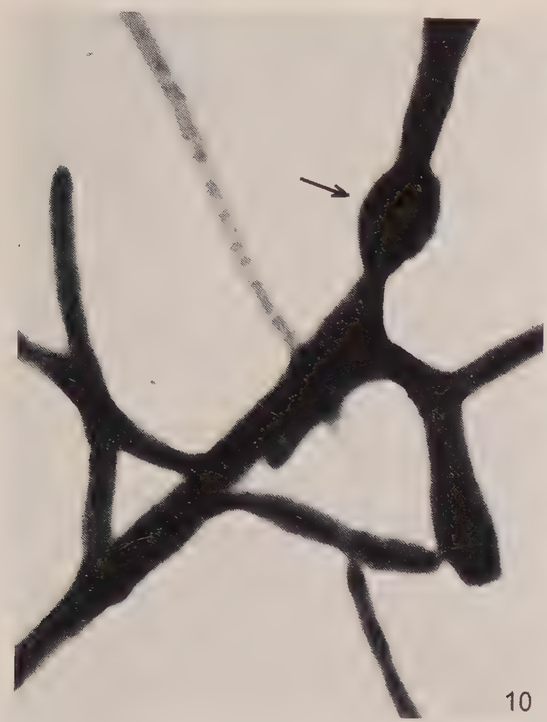
P. B. Dickenson and K. D. Macdonald—Initial cells in *Streptomyces* spp. Plate 2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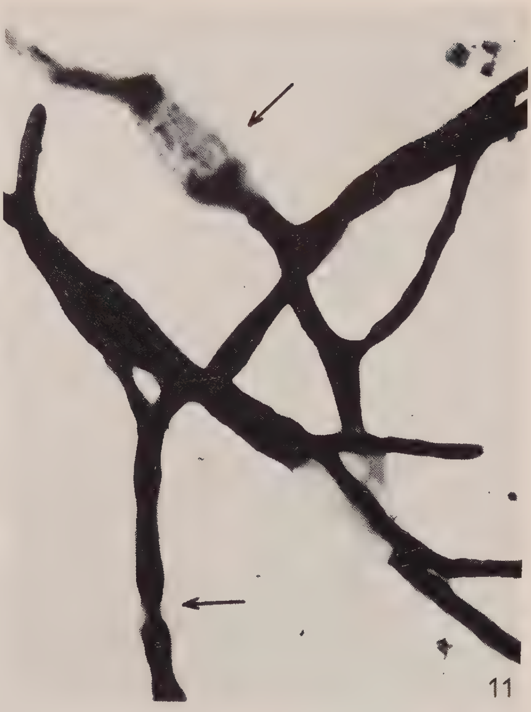
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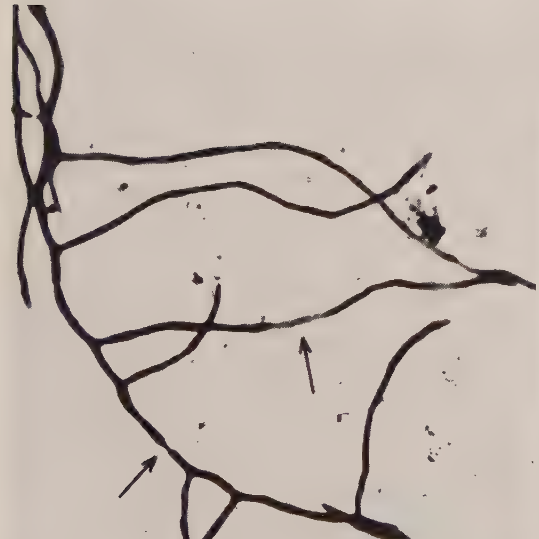
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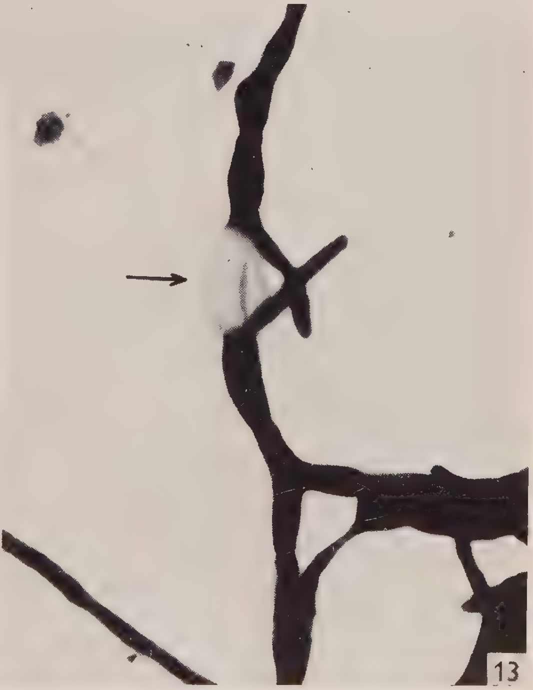
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P. B. Dickenson and K. D. Macdonald—Initial cells in *Streptomyces* spp. Plate 3

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The Statistical Analysis of Plant Virus Assays: a Transformation to Include Lesion Numbers with Small Means

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SUMMARY: The numbers of local lesions (x) produced by different leaves when inoculated with the same virus preparation deviate greatly from a normal distribution, and the standard errors of x's depend on their magnitude. Before customary statistical analyses are applicable to results of infectivity assays, x's must be suitably transformed. When mean values of x are greater than about 10, the transformation $z=\log_{10}(x+c)$ (where c is a constant), is satisfactory but inapplicable with smaller numbers. In some work the use of poorly infective inocula is unavoidable, and to allow statistical analysis of results in such work a new transformation $z=\log_{10}\frac{1}{2}[x+c+\sqrt{(x^2+2cx)}]$ is derived. This operates satisfactorily when mean numbers of x are greater than about 1·5. The results of the two transformations converge as x increases. Values of z are tabulated to make the use of the new transformations as quick as that of the other transformation.

Numbers of local lesions produced by inoculating plant viruses to leaves of host plants cannot be used directly in routine statistical analyses, such as analysis of variance, because their standard errors depend on their magnitude and their frequency distribution deviates very considerably from normal. Therefore, the transformation

$$z=\log_{10}(x+c) \tag{1}$$

was proposed (Kleczkowski, 1949), in which x's are numbers of lesions on individual leaves or half-leaves (whichever is used as a unit) and c is a constant whose value usually lies between 5 and 15. The values of z are approximately normally distributed with standard errors independent of their magnitude, when values of x are not too small. An exact limiting value cannot be given, for it varies with experimental conditions and with different virus-host systems. However, the transformation (1) usually makes the standard error of z constant when the mean number of lesions is greater than about 10, but the standard error begins to decrease as the mean decreases between 10 and 0. Thus transformation (1) can only be used safely when the mean numbers of lesions given by individual inocula are greater than 10 and never when they are smaller than 5.

When infectivities of several preparations of a virus are to be compared, a test should be so designed that as many sources of variation as possible are eliminated. This is done by suitably allotting leaves or half-leaves for different inocula. When at least one of the tested inocula gives a mean number of lesions too small for the transformation (1) to be applied, the usual analysis of variance of the whole experiment using the transformation (1) may give false results. As in many kinds of problems the use of inocula that form only a few lesions per leaf cannot be avoided, a transformation that would still be applicable is needed if the results are to be analysed statistically; this paper describes the derivation and the use of such a transformation.

Derivation

The relationship between the means ($\bar{s}$) of estimated standard errors (s) of numbers of lesions (x) and of the means of numbers of lesions (m) is given by the regression equation

$$\bar{s}=b(m+c), \tag{2}$$

where b is the regression coefficient and c is a constant identical with that of the transformation (1) (Kleczkowski, 1949). The relationship between the standard error on the mean number of lesions is thus given by the equation

$$s=\beta(m+c), \tag{3}$$

the value of β being slightly greater than that of b of equation (2) because mean values of standard errors are smaller than square roots of mean values of variances; the ratio of β to b depends on the size of samples from which variances and standard errors are computed, and approaches 1 as the size of the sample increases. Thus, the transformation, that could be expected to make the standard error of transformed values constant and approximately equal to 1, is

$$y=\int\frac{dx}{\beta(x+c)}=\frac{1}{\beta}\log_e(x+c). \tag{4}$$

The use of the transformation (1) instead of (4) would make the standard error of z approximately equal to $0{\cdot}4343\beta$.

The values of z given by the transformation were, indeed, found to have a standard error independent of their magnitude and were approximately normally distributed (Kleczkowski, 1949). However, the standard error can be considered to be the linear function of the mean given by (3) only when the mean is not smaller than about 10, and, as the mean becomes smaller, the value of the standard error deviates appreciably from the straight line (3) and approaches 0 as the mean approaches 0. Moreover, discontinuity of the transformed variable (x's being integers) becomes more pronounced as the variate decreases, and this may eventually upset conditions that assume an approximately normal, and thus continuous, distribution of the transformed values. However, disregarding for the moment any possible effects of discontinuity, the deviation of the standard error from the line (3) alone makes the transformation (1) or (4) unsuitable when the deviation becomes appreciable. A transformation was, therefore, derived which is based on the assumption that the standard error of x follows a curve that starts at 0 when $m=0$ and then approaches asymptotically the straight line given by (3) as m increases. A curve that fulfils these conditions is the hyperbola

$$s=\beta\sqrt{[(m+c)^2-c^2]}. \tag{5}$$

Assuming (5) to be true, the transformation that may be expected to equalize the standard error would be

$$y=\int\frac{dx}{\beta\sqrt{[(x+c)^2-c^2]}}=\frac{1}{\beta}\log_e[x+c+\sqrt{(x^2+2cx)}]. \tag{6}$$

The variance of the values of y would be expected to be approximately equal to 1. In practice the transformation

$$z=\log_{10}\tfrac{1}{2}[x+c+\sqrt{(x^2+2cx)}] \tag{7}$$

is more convenient. As x increases, the value of z given by (7) approaches that given by (1), so that for sufficiently high values of x the values of z can be computed more simply using (1) than using (7). The standard error of z would be expected to be approximately $0{\cdot}4343\beta$.

There is no evidence that (5) does truly express the dependence of the standard error of numbers of lesions on their means, but the standard errors probably follow a curve not very different from (5); in the absence of anything better, it was assumed to be true and the transformation (7) was applied to experimental data to see whether the results would be satisfactory.

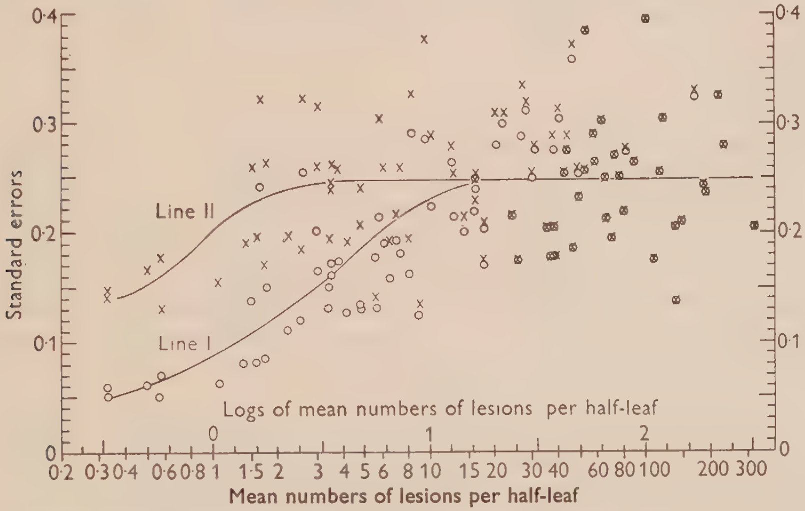

Fig. 1. Relationship between standard errors of transformed lesion numbers and the means of the numbers. Transformation $z=\log_{10}(x+c)$: points ○ and line I. Transformation $z=\log_{10}\frac{1}{2}[x+c+\sqrt{(x^2+2cx)}]$: points × and line II.

Application to experimental data

Fig. 1 compares results of transformations (1) and (7) of numbers of lesions produced by tobacco mosaic virus on leaves of *Nicotiana glutinosa* in several separate experiments. In each experiment six different inocula containing virus at different dilutions, or at different levels of activity remaining after exposure to different amounts of ultraviolet radiation, were inoculated on to half-leaves of six plants. Each plant had six inoculable leaves. The treatments were so distributed that each inoculum occurred twice on each plant and on each leaf position. There were, therefore, 12 replications of each treatment (inoculum).

The constant c was taken arbitrarily as 5 for all the transformations, for previous experience showed that this value lies within the range of values that usually give satisfactory results. The standard errors of the transforms were computed separately for each treatment without making adjustments to eliminate variation between plants and leaf positions. It is important that the transformation should give satisfactory results even when variation between plants and leaf positions cannot be eliminated, because some experiments can only be designed so that variations between 'blocks' comprising a few plants or between leaf 'levels' comprising a few leaf positions, can be eliminated, and in some experiments even this cannot be done.

Fig. 1 shows standard errors of the transforms (1) and (7) plotted against the logarithms of mean numbers of lesions per half-leaf. To make inspection of the distribution of the standard errors easier, lines I and II were passed approximately through mean values of standard errors of the transforms (1) and (7), respectively, in different regions of the logarithms of mean numbers of lesions. The course of the lines shows that standard errors of the transforms (1) and (7) can be considered independent of their magnitude only when the mean numbers of lesions per half-leaf are not smaller than about 10 and 1·5, respectively.

In the region where the standard errors of the transforms can be considered independent of the mean numbers of lesions, the mean value of the standard errors was about 0·25. If the distribution of numbers of lesions does approximately correspond with the assumptions on which the transformations are based, the mean value of the standard errors of the transforms should be near the value of $0{\cdot}4343b$, where b is the coefficient of regression of standard errors of untransformed numbers of lesions, whose means are not smaller than 10, on the means. The value of b in most experiments was found to be about 0·5, so that the value of $0{\cdot}4343b$ is 0·22 which is, indeed, near to the mean value of the standard errors of the transforms.

The conclusion is that the transformation (1) can be used only when mean numbers of lesions given by different treatments are not smaller than about 10, whereas the corresponding limit for the transformation (7) is about 1·5.

The transformation (7) is more laborious than (1), which is simply read from a logarithmic table or from a slide rule. To make the transformation (7) as quick to use as (1), Table 1 is appended. As only two first decimal figures need be used, the table gives the values of the transform (7) for several values of the constant c only as far as two first decimal figures of the transform (7) differ from those of the transform (1). For all higher values of x the transformation (1) would give the same results as (7) and so can be used instead of (7).

An important condition before either transformation (7) or (1) can be applied is that the test plants should not contain any exceptionally resistant to infection. Including a plant that produces very few lesions increases the ranges of variability of numbers of lesions produced by all treatments. However, the increase will be disproportionately greater with treatments that produce many lesions on the other plants than with treatments that produce few lesions. Consequently, the transformation may fail to equalize the standard errors.

Table 1. *The values of* $z=\log_{10}\frac{1}{2}[x+c+\sqrt{(x^2+2cx)}]$

For values of x higher than those given in this table $z=\log_{10}(x+c)$.

x	z	x	z	x	z	x	z	x	z	x	z	x	z	x	z
$c=3$		$c=5$		$c=6$		$c=10$		$c=10$		$c=15$		$c=20$		$c=20$	
0	0·18	6	1·02	21	1·43	0	0·70	43	1·72	33	1·67	7	1·35	50	1·84
1	0·52	7	1·06	22	1·44	1	0·89	44	1·73	34	1·68	8	1·38	51	1·84
2	0·65	8	1·10	23	1·46	2	0·97	45	1·74	35	1·69	9	1·40	52	1·85
3	0·75	9	1·13	24	1·47	3	1·03	46	1·75	36	1·70	10	1·42	53	1·86
4	0·82	10	1·16	25	1·49	4	1·08	47	1·75	37	1·71	11	1·44	54	1·86
5	0·89	11	1·19	26	1·50	5	1·12	48	1·76	38	1·72	12	1·46	55	1·87
6	0·94	12	1·22	27	1·52	6	1·15	49	1·77	39	1·72	13	1·47	56	1·87
7	0·99	13	1·25	28	1·53	7	1·19			40	1·73	14	1·49	57	1·88
8	1·03	14	1·27			8	1·22	$c=15$		41	1·74	15	1·50	58	1·89
9	1·07	15	1·29	$c=7$		9	1·25			42	1·75	16	1·52	59	1·89
10	1·11	16	1·32			10	1·27	0	0·88	43	1·76	17	1·53	60	1·90
11	1·14	17	1·34	0	0·54	11	1·30	1	1·03	44	1·76	18	1·55	61	1·90
12	1·17	18	1·36	1	0·77	12	1·32	2	1·10	45	1·77	19	1·56	62	1·91
13	1·20	19	1·38	2	0·87	13	1·34	3	1·15	46	1·78	20	1·57	63	1·91
		20	1·39	3	0·93	14	1·36	4	1·19	47	1·79	21	1·59	64	1·92
$c=4$		21	1·41	4	0·99	15	1·38	5	1·22	48	1·79	22	1·60	65	1·92
		22	1·43	5	1·04	16	1·40	6	1·25	49	1·80	23	1·61	66	1·93
0	0·30	23	1·44	6	1·08	17	1·42	7	1·28	50	1·81	24	1·62	67	1·93
1	0·60	24	1·46	7	1·12	18	1·43	8	1·31	51	1·81	25	1·63	68	1·94
2	0·72			8	1·15	19	1·45	9	1·33	52	1·82	26	1·64	69	1·94
3	0·80	$c=6$		9	1·18	20	1·47	10	1·35	53	1·83	27	1·65	70	1·95
4	0·87			10	1·21	21	1·48	11	1·37	54	1·83	28	1·66	71	1·95
5	0·93	0	0·48	11	1·24	22	1·49	12	1·39	55	1·84	29	1·67	72	1·96
6	0·98	1	0·72	12	1·26	23	1·51	13	1·41	56	1·85	30	1·68	73	1·96
7	1·03	2	0·82	13	1·29	24	1·52	14	1·43	57	1·85	31	1·69	74	1·97
8	1·07	3	0·89	14	1·31	25	1·54	15	1·45	58	1·86	32	1·70	75	1·97
9	1·10	4	0·95	15	1·33	26	1·55	16	1·46	59	1·87	33	1·71	76	1·98
10	1·14	5	1·00	16	1·35	27	1·56	17	1·48	60	1·87	34	1·72	77	1·98
11	1·17	6	1·05	17	1·37	28	1·57	18	1·49	61	1·88	35	1·73	78	1·99
12	1·20	7	1·09	18	1·39	29	1·58	19	1·51	62	1·88	36	1·73	79	1·99
13	1·22	8	1·13	19	1·41	30	1·60	20	1·52	63	1·89	37	1·74	80	2·00
14	1·25	9	1·16	20	1·42	31	1·61	21	1·54	64	1·89	38	1·75	81	2·00
15	1·27	10	1·19	21	1·44	32	1·62	22	1·55	65	1·90	39	1·76	82	2·01
16	1·30	11	1·22	22	1·46	33	1·63	23	1·56			40	1·77	83	2·01
		12	1·24	23	1·47	34	1·64	24	1·57	$c=20$		41	1·77	84	2·01
$c=5$		13	1·27	24	1·49	35	1·65	25	1·59			42	1·78	85	2·02
		14	1·29	25	1·50	36	1·66	26	1·60	0	1·00	43	1·79	86	2·02
0	0·40	15	1·31	26	1·51	37	1·67	27	1·61	1	1·14	44	1·80	87	2·03
1	0·67	16	1·33	27	1·53	38	1·68	28	1·62	2	1·19	45	1·80	88	2·03
2	0·78	17	1·35	28	1·54	39	1·69	29	1·63	3	1·24	46	1·81	89	2·03
3	0·85	18	1·37	29	1·55	40	1·70	30	1·64	4	1·27	47	1·82	90	2·04
4	0·92	19	1·39	30	1·57	41	1·70	31	1·65	5	1·30	48	1·82	91	2·04
5	0·97	20	1·41	31	1·58	42	1·71	32	1·66	6	1·33	49	1·83	92	2·05

To choose the value of the constant c, standard errors of untransformed numbers of lesions obtained with different treatments, whose means are not smaller than about 10, are plotted against the means, and a regression line is roughly passed through the plotted points. The value of c is the distance between the origin and the point of intersection of the regression line with the axis of abscissae, which should be on the negative part of the axis. The choice of c need not be based on experimental results that are actually analysed. It can be based on results of other experiments made with the same virus-host system in similar conditions, or the value of c may be found from a special experiment. No great accuracy is usually required in choosing the value of c, for any value from a rather wide range gives satisfactory results. For example, any value between 5 and 10 is usually satisfactory with numbers of lesions produced by tobacco mosaic virus or tomato bushy stunt virus on leaves of *Nicotiana glutinosa*. As the choice of c is to some extent arbitrary and need not be based on any information obtained from the experiment whose results are actually analysed, obviously it does not cause a loss of a degree of freedom in the final analysis of the results.

If an experiment is done only to see whether different inocula have different infectivities, or whether plants differ in their susceptibility to a given virus, the usual analysis of variance followed by a customary test of significance is simply carried out using the transforms (7). However, if an experiment is done to test a hypothesis that predicts that a treatment reduces activity of a virus preparation to a certain proportion of the original activity, special methods of analysis are necessary. A method that can be used for such tests was described for the transformation (1) (Kleczkowski, 1953). The transformation (7) requires a modification of the method.

Testing results obtained for compatibility with hypotheses

Let us assume, as previously (Kleczkowski, 1953), that a hypothesis is tested which predicts that a treatment reduces activity of a virus preparation to a proportion p, which is a function f of a variable ψ and an adjustable constant k, so that

$$p = f(\psi, k). \tag{8}$$

To test this, inocula, some containing variously diluted untreated virus and others containing virus that has been subjected to the treatment under test, are inoculated to leaves (or half-leaves) of test plants, distributing the different inocula so that analysis of variance can subsequently be applied.

It is usually possible, by suitably adjusting the values of three constants, N, ξ and λ, to express the relationship between the numbers of lesions (Y) and the concentration of a virus in the inoculum (h) by the equation

$$Y = \frac{N}{\lambda\sqrt{2\pi}} \int_{-\infty}^{u} \exp\left\{-\tfrac{1}{2}\left(\frac{r-\xi}{\lambda}\right)^2\right\} dr, \tag{9}$$

where $u = \log_{10} h$ (Kleczkowski, 1950, 1953). If the virus preparation has been subjected to the treatment under test and its activity is expected to be altered by the factor p of equation (8), $u = \log_{10} ph$.

The value of Y of equation (9) is considered to be the postulated mean number of lesions expected to be formed by a given inoculum. Now, in the transformation (7) logarithms are taken of experimental values of

$$v = \tfrac{1}{2}[x + c + \sqrt{(x^2 + 2cx)}].$$

The mean value of v (say $\bar{v}$) is half the sum of the mean value of $(x+c)$, which is $(m+c)$, and of the mean value of $t=\sqrt{(x^2+2cx)}$. To find the mean value of t (say $\bar{t}$) use is made of the fact that $\text{var}\,(t)=E(t^2)-\bar{t}^2$. Therefore

$$\text{var}\,(t)=E(x^2+2cx)-\bar{t}^2=E(x^2)+2cm-\bar{t}^2=\text{var}\,(x)+m^2+2cm-\bar{t}^2.$$

Thus $\bar{t}=\sqrt{[m^2+2cm-\text{var}\,(t)+\text{var}\,(x)]}$.

The variance of x is given by (5) and equals approximately $\beta^2(m^2+2cm)$. To obtain the variance of t we notice that $dt/dx=(x+c)/\sqrt{(x^2+2cx)}$, which means that t is obtained from x by the transformation

$$t=\int\frac{(x+c)dx}{\sqrt{(x^2+2cx)}}. \tag{10}$$

As the transformation (6) gives the values of y with a variance approximately equal to 1, the transformation (10) will make the variance of t approximately equal to $\beta^2(m+c)^2$. It can be assumed, therefore, that

$$\text{var}\,(t)-\text{var}\,(x)=\beta^2c^2, \tag{11}$$

and, therefore, $\bar{t}=\sqrt{(m^2+2cm-\beta^2c^2)}$, so that $\bar{v}=\frac{1}{2}[m+c+\sqrt{(m^2+2cm-\beta^2c^2)}]$. Thus, if Y of equation (9) is the postulated mean number of lesions, the postulated value of $\bar{v}$ will be

$$\bar{V}=\tfrac{1}{2}[Y+c+\sqrt{(Y^2+2cY-\beta^2c^2)}]. \tag{12}$$

The values of $\bar{z}$, i.e. the means of experimental values of z of the transformation (7), are decimal logarithms of geometric means of v. Thus, to be compared with the values of $\bar{z}$, the postulated arithmetic means of the values of v must be converted into decimal logarithms of geometric means. Let $\bar{Z}$ be the logarithm of the postulated geometric mean of v. Assuming the values of z to be normally distributed with the mean $\bar{Z}$ and variance σ^2, the postulated arithmetic mean of $v=10^z$ is

$$\bar{V}=\frac{1}{\sigma\sqrt{2\pi}}\int_{-\infty}^{\infty}10^z\exp\left\{-\tfrac{1}{2}\left(\frac{z-\bar{Z}}{\sigma}\right)^2\right\}dz=10^{\bar{Z}}\,e^{\frac{1}{2}(2\cdot3\sigma)^2},$$

so that

$$\bar{Z}=\log_{10}\bar{V}-1\cdot15\sigma^2=\log_{10}\tfrac{1}{2}[Y+c+\sqrt{(Y^2+2cY-\beta^2c^2)}]-1\cdot15\sigma^2. \tag{13}$$

The estimate of variance of a single value of z, say s^2, obtained from analysis of variance of the results, can be used as σ^2. The value of β^2 can be obtained from s^2 if it is remembered that s^2 is approximately equal to $(0\cdot4343\beta)^2$, so that β^2 can be taken as equal to $5\cdot29s^2$. Substituting these values into (13) we obtain

$$\bar{Z}=\log_{10}\tfrac{1}{2}[Y+c+\sqrt{(Y^2+2cY-5\cdot29s^2c^2)}]-1\cdot15s^2. \tag{14}$$

The value of Y is given by (9) and c is the constant of the transformation (7).

To avoid obtaining false values of Z by equation (14) the value of c should be more carefully chosen than when experimental results are merely tested for significance of differences between treatments. Although the choice of c need not be based on the data of the experiment whose results are actually analysed, a check should be made to see whether the chosen value of c does indeed correspond with the conditions of the experiment. A convenient method of doing this is based on computing variances of untransformed numbers of lesions (x) and also of the values $t=\sqrt{(x^2+2cx)}$ separately for each treatment (inoculum), without making any adjustments to eliminate any known sources of variation such as between plants or leaf positions. The differences between variances of t and x should not tend to increase or decrease with the increasing means of x and should on average be equal to $\beta_1 c^2$ (see (11)). (β_1 may be greater than β of equations (12) and (13), because the latter may be based on results of analysis of variance, which would be expected to decrease the value of β.) The value of β_1 can approximately be obtained by plotting standard errors of x's for different treatments, whose means are not smaller than about 10, against the means and passing roughly a regression line through the plotted points. The value of β_1 can be assumed approximately equal to the slope of the line. In fact it should be slightly greater than the slope, for the same reason that β of (3) is greater than b of (2), but the difference decreases with increasing size of samples and can be neglected when the number of replications is 10 or more. With tobacco mosaic virus inoculated to leaves of *Nicotiana glutinosa* in our conditions the value of β_1 was usually about 0·5 and the value of c about 6.

Further procedure is similar to that previously described for the transformation (1) (Kleczkowski, 1953). The values of the constants k, N, ξ and λ of equations (8) and (9) are adjusted by the method of least squares, i.e. by minimizing the value of $\Sigma(\bar{z}_i - \bar{Z}_i)^2$, where $\bar{z}_i$'s are the mean values of z obtained with different inocula and $\bar{Z}_i$'s are the corresponding values of $\bar{Z}$ of equation (14). If each $\bar{z}_i$ is a mean of n values of z, $n\Sigma(\bar{z}_i - \bar{Z})^2/W$, where W is the number of degrees of freedom equal to the number of inocula minus the number of adjustable constants of equations (8) and (9), should be an estimate of the variance of a single value of z independently of that given by s^2, obtained by the customary procedure of the analysis of variance. If the hypothesis on which equation (8) is based is true, the two estimates should be compatible, and this can be tested by the variance ratio test.

The values of the constants of equations (8) and (9) can be adjusted as described previously (Kleczkowski, 1953). If it becomes necessary to use 'normal' equations, the partial derivatives of $\bar{Z}$'s can be obtained from those previously listed for the procedure based on the transformation (1) by substituting $[Y+c+\sqrt{(Y^2+2cY-5{\cdot}29s^2c^2)}]$ for $(Y+c)$ and multiplying by $(1+(Y+c)/\sqrt{(Y^2+2cY-5{\cdot}29s^2c^2)})$.

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Pathogenic Strains of *Fusarium oxysporum* Fr. Distinguished by their Differential Tolerance to Inhibition by various Actinomycetes

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SUMMARY: Cultures of sixteen different soil actinomycetes were tested for their ability to inhibit the growth of eight pathogenic strains of *Fusarium oxysporum* Fr. on agar media. Three of the actinomycetes did not inhibit growth, and nine inhibited growth of all strains equally, but the other four actinomycetes consistently inhibited the growth of individual strains to different extents. The differences provide an *in vitro* test which distinguishes between certain pathogenic strains of *Fusarium* that are otherwise indistinguishable in culture.

Fusarium oxysporum Fr. is an Imperfect fungus comprising many pathogenic strains which are indistinguishable in culture. The complex system of classification proposed by Wollenweber & Reinking (1935), in which many species were described, was simplified by Snyder & Hansen (1940), who grouped all the members under the name *F. oxysporum* Fr. The many forms of this species cause wilt diseases of several important crops, e.g. banana, tomato, flax and peas. Each pathogenic strain is designated *forma specialis* according to its specific pathogenicity towards a particular host. Within these strains, physiologic races occur, the races differing in their abilities to cause disease in different varieties of the host. Eight members of the species *F. oxysporum* Fr. were grown in culture with sixteen actinomycetes to see whether the actinomycetes affected the growth of the fusarium strains and, if so, whether the inhibition was specific and in any way correlated with the pathogenicity of the fusarium strains.

METHODS

The eight fusarium strains used, with their laboratory numbers and origins, were as follows: 71B, *Fusarium oxysporum* f. *pisi* race 1, from wilted peas; 72B, *F. oxysporum* f. *pisi* race 2, from 'near-wilted' peas; 611A, *F. oxysporum* f. *pisi* race 3A, from wilted peas (Buxton, 1955); 612A, *F. oxysporum* f. *pisi*, from wilted peas, equivalent in pathogenicity to 71B; 51A, *F. oxysporum* F. (syn. *F. oxysporum* Fr. var. *redolens* (Wr.), n.comb. Gordon, from wilted peas; F1, *F. oxysporum* f. *gladioli*, yellowing strain from Gladiolus; F2, *F. oxysporum* f. *gladioli*, yellowing and rotting strain from Gladiolus; 61A, *F. oxysporum* Fr., non-pathogenic to peas, isolated from soil.

Sixteen actinomycetes, isolated from soil, were tested against the eight fusaria. The tests were made on medium constituted as follows: (g./l. water; $NaNO_2$, 2·0; K_2HPO_4, 1·0; $MgSO_4.7H_2O$, 0·5; KCl, 0·5; $FeSO_4.7H_2O$, 0·01;

Table 1. *Differential tolerance of eight strains of* Fusarium oxysporum *to inhibition by sixteen actinomycetes as shown by the distances (mm.) grown towards the actinomycete colonies*

Isolate number	*Fusarium* species	Origin	Actinomycetes															
			A 1	A 2	A 3	A 4	A 5	A 6	A 9	A 14	A 16	A 17	A 19	A 26	A 27	A 28	A 29	A 30
			Distances to nearest 0·5 mm. Low numbers represent severe inhibition.															
71 B	*oxysporum* f. *pisi* race 1	Wilted peas, U.S.A.	18·0	25·0	17·0	8·0	13·0	24·5	22·5	15·0	10·5	13·0	19·0	22·0	24·0	17·0	23·0	23·5
72 B	*oxysporum* f. *pisi* race 2	Wilted peas, U.S.A.	16·5	24·0	14·5	7·5	12·0	23·5	22·0	10·5	11·0	9·0	25·0	15·0	12·5	18·0	18·0	16·0
611 A	*oxysporum* f. *pisi* race 3	Wilted peas, England	19·0	21·0	18·0	8·5	22·5	25·5	22·0	14·0	17·0	13·0	22·5	19·0	19·0	20·0	24·0	23·5
612 A	*oxysporum* f. *pisi* race 1	Wilted peas, England	19·5	27·5	19·0	10·0	16·0	26·0	20·0	11·0	13·0	12·0	20·5	13·5	22·0	14·0	24·0	20·0
51 A	*oxysporum* var. *redolens*	Wilted peas, England	18·5	21·5	16·5	9·0	23·5	26·0	23·5	15·0	20·0	11·0	20·0	21·0	21·0	20·0	24·5	18·0
F 1	*oxysporum* f. *gladioli*	Yellowed gladiolus, England	19·5	24·0	17·0	9·5	19·0	28·0	20·5	13·0	18·0	11·5	20·0	22·0	21·0	21·5	25·0	23·5
F 2	*oxysporum* f. *gladioli*	Yellowed gladiolus, England	20·0	22·5	16·5	9·5	18·0	25·0	22·0	13·0	16·0	11·5	20·5	18·0	20·0	18·5	23·0	21·5
61 A	*oxysporum*	Soil, England	18·5	24·0	21·0	10·5	18·5	25·5	22·0	13·0	20·0	11·0	20·5	17·0	23·5	18·5	21·5	22·0

soluble starch, 10·0; Bacto-tryptone, 1·0; agar, 15; pH adjusted to 7·0 with a concentrated solution of NaOH.

A single streak of an actinomycete was made on a Petri dish containing 10 ml. of the medium; this was then incubated for 5 days at 25°. A streak of a fusarium (as spore suspensions of uniform density) was then made parallel to the actinomycete colony and 30 mm. distance from it. The plates were incubated for 5 more days before the growth of the fusarium towards the actinomycete was measured, these times having been found optimal for showing inhibition.

RESULTS

Three of the actinomycete colonies (A4, A14, A17) strongly inhibited all eight fusaria, six (A1, A3, A9, A19, A28, A30) inhibited all less strongly, and three (A2, A6, A29) allowed them to grow normally, so that the fusarium finally enveloped the actinomycete colony. Four of the actinomycetes (A5, A16, A26, A27) inhibited different fusaria to different extents. Control streaks of the fusaria grew beyond the 30 mm. lines after 6 days. Strongly inhibited fusarium strains had advancing edges which consisted entirely of tightly packed stunted hyphae, whereas partly inhibited strains had marginal hyphae which branched subnormally. The inhibition shown by actinomycete A5 (Table 1), identified as a strain of *Streptomyces albidoflavus* (Rossi Doria) Waksman & Henrici, towards strains 72B (*F. oxysporum* f. *pisi*, race 2) and 611A (*F. oxysporum* f. *pisi*, race 3A) is shown in Pl. 1, fig. 1. Zones of inhibition by the same actinomycete on dishes seeded with fusaria are shown in Pl. 1, fig. 2. Culture filtrates of *S. albidoflavus* grown in liquid starch-tryptone medium (constituted as above) were tested against the fusarium strains. Porcelain cylinders containing 0·1 ml. of the filtrate were placed on the surface of dishes containing 10 ml. of starch tryptone agar medium seeded with spores of fusaria. Inhibition differences by the filtrates were analogous to those observed in the streak method and are shown in Pl. 1, fig. 3.

DISCUSSION

There is nothing new in the fact that growth products from actinomycetes should inhibit the growth of fusaria in culture or in the soil. Kublanovskaya (1952) suppressed wilt of cotton, caused by *Fusarium vasinfectum*, and Lachange & Perrault (1953) wilt of flax, caused by *F. lini*, by adding actinomycetes to infected soil. Neither variations in the susceptibility of different pathogenic strains of *Fusarium* nor indeed variations in any strains of wild-type fungus to the action of actinomycete inhibitors, have been previously recorded. Mitchison (1951), however, found isolates of *Mycobacterium tuberculosis* with different abilities to tolerate streptomycin, and Katznelson & Sutton (1953) detected different reactions of strains of *Xanthomonas translucens* to bacteriophage. Such variations are not unexpected, for the ability to grow in the presence of substances which inhibit other organisms in any natural environment has obvious survival value. The plasticity afforded by variations in reaction by different strains to actinomycete inhibition would be important to heterogeneous soil populations of *Fusarium* spp. The main immediate value of these differences is that they provide a simple *in vitro* method of distinguishing between isolates of *F. oxysporum* that otherwise are distinguishable only by laborious infection tests. Previously, during our experiments concerned with the genetics of pathogenicity in *F. oxysporum*, the only way of distinguishing *in vitro* between isolates used in combined infections was to produce biochemical or morphological mutants by such treatments as irradiation with ultraviolet light. We have found that mutants produced by these treatments are, however, usually decreased in their infectivity. A natural difference in ability to tolerate growth products of an actinomycete provides a test that avoids the complications introduced by artificial mutagenic methods, for the genetical 'markers' concerned with tolerance to inhibition are related to wild-type pathogenicity.

We wish to thank Dr F. A. Skinner of the Soil Microbiology Department, Rothamsted, for providing the actinomycete cultures.

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EXPLANATION OF PLATE

Fig. 1. Differences in tolerance shown by *Fusarium oxysporum* f. *pisi*, race 2 (left) and *F. oxysporum* f. *pisi*, race 3A (right) towards *Streptomyces albidoflavus*. (× ⅔.)

Fig. 2. Zones of inhibition of *F. oxysporum* f. *pisi*, race 2 (left) and *F. oxysporum* f. *pisi*, race 3 (right) caused by *S. albidoflavus*. (× ⅔.)

Fig. 3. Effect of a culture filtrate of *S. albidoflavus* on three strains of *F. oxysporum*. *a*, *F. oxysporum* f. *pisi*, race 2: strongly inhibited. (× ⅔.) *b*, *F. oxysporum* f. *gladioli*, strain F1: mildly inhibited. (× ⅔.)

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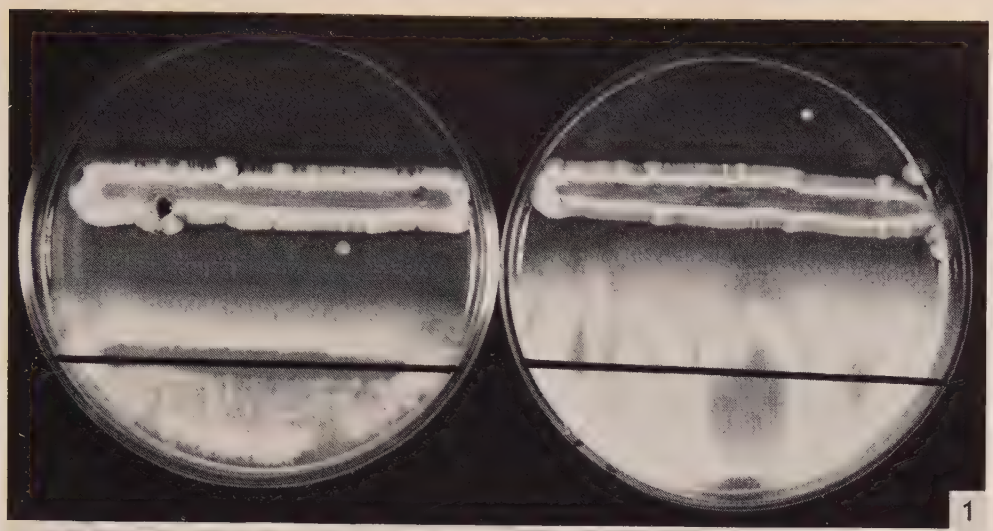
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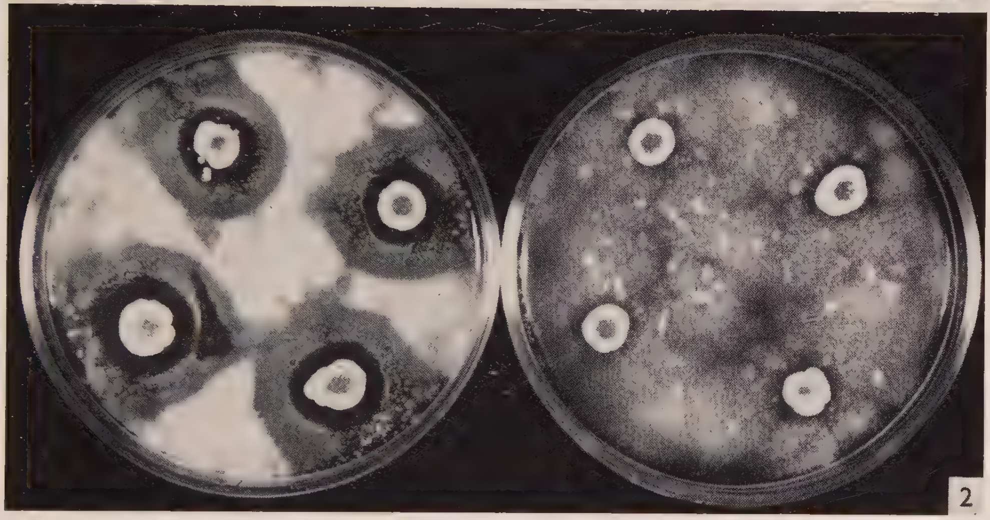
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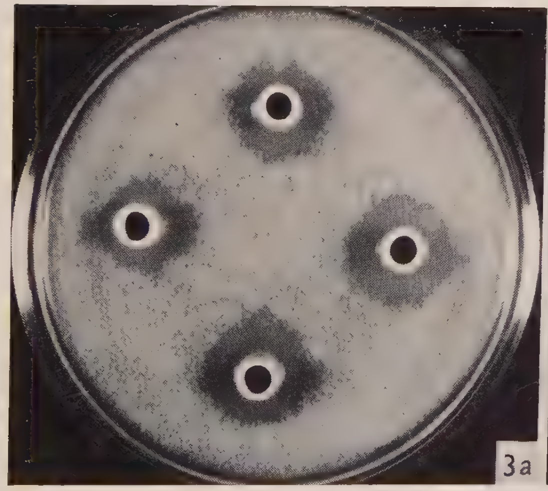
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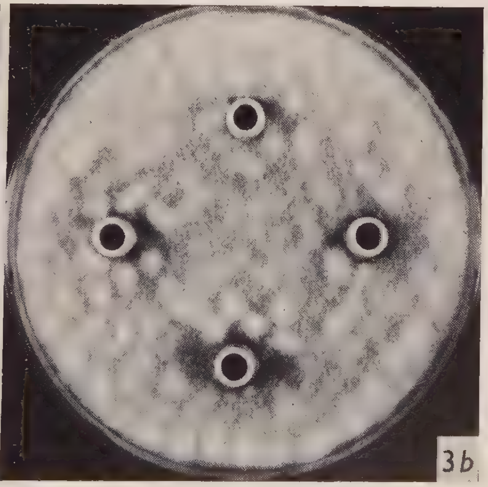
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E. W. BUXTON AND M. G. RICHARDS—INHIBITORY DISTINCTION BY ACTINOMYCETES. PLATE 1

(*Facing p.* 102)

HARRIS, W. J. (1955). *J. gen. Microbiol.* **13**, 103–4.

Demonstration of the Existence of Spore Sacs around Resting Spores of *Bacillus subtilis* and *Bacillus globigii*

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Up to the present time, it has not been shown conclusively that germinating spores possess sacs before germination commences. However, in the course of an electron microscope study of the spores of *Bacillus subtilis* and *B. globigii* evidence has been obtained to show the existence of well-defined sacs around resting spores.

Complete spore sacs, similar to those vacated by germinated spores, have been stripped from a proportion of freeze-dried spores subjected to high gas pressure subsequently quickly released. The method employed was somewhat similar to that used by Fraser (1951) for bursting bacteria, but the pressure of the gas (nitrogen) was higher (1500 lb./in.^{2}) and the release almost instantaneous. This was achieved by the rupture of a metal diaphragm incorporated in the cylinder containing the spores (method devised by Mr J. Edwards.)

The micrographs obtained also illustrate distinct differences in the physical characteristics of the spore sacs of *Bacillus subtilis* and *B. globigii* (cf. Lamanna, 1940). Pl. 1, fig. 1 shows empty spore sacs of *B. subtilis* vacated by germinated spores, and Pl. 1, fig. 2, a typical example of a sac removed by quick release of gas pressure. In both cases the sac appears to be a hard structure strengthened in the polar regions and having a central region of weakness where rupture always occurs. The spore sac of *B. globigii*, however, appears to be a soft flimsy structure with a pronounced weakness at one pole where rupture always takes place, whether during germination (Pl. 1, fig. 3) or as a result of quick release of gas pressure (Pl. 1, fig. 4). Evidence obtained indicates that after polar rupture by the latter process in *B. globigii* spores, sac removal is caused by fragmental tearing (Pl. 1, fig. 5).

Acknowledgement is made to the Chief Scientist, Ministry of Supply, for permission to publish this note.

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EXPLANATION OF PLATE

Fig. 1. Spore sacs of *B. subtilis* vacated by germinated spores; ×10,000.

Fig. 2. Field showing spore sac of *B. subtilis* removed by quick release of gas pressure; ×10,000.

Fig. 3. Germinated spore of *B. globigii*; ×10,000.

Fig. 4. Spore sac of *B. globigii* removed by quick release of gas pressure; ×10,000.

Fig. 5. Illustration of the mechanism of spore sac removal in *B. globigii* by quick release of gas pressure; ×10,000.

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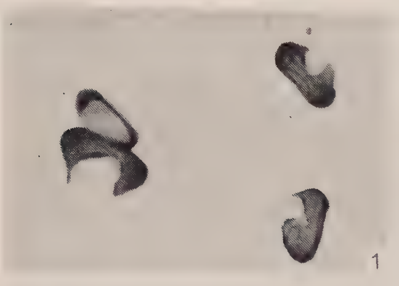
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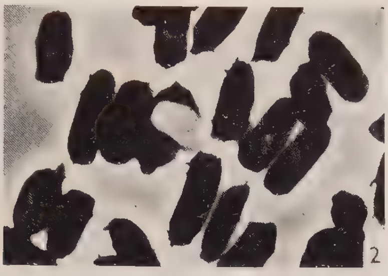
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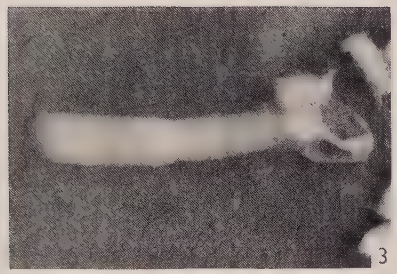
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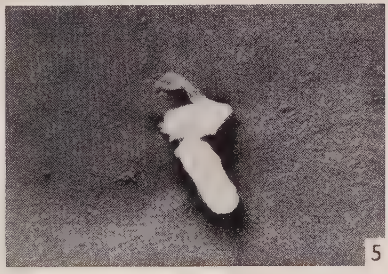
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W. J. HARRIS—EXISTENCE OF SPORE SACS IN *BACILLUS SUBTILIS* AND *BACILLUS GLOBIGII* PLATE 1

(*Facing p.* 104)

Tomcsik, J. & Grace, J. B. (1955). *J. gen. Microbiol.* **13**, 105–110.

Bacterial Cell Walls as Revealed by the Specific Cell-wall Reaction and by Direct Staining with Alcian Blue

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SUMMARY: Isolated cell walls of strains of *Bacillus*, *Micrococcus* and *Caryophanon* species obtained by mechanical disintegration, and in some instances by the action of trypsin, gave a specific cell-wall reaction, visible in the phase-contrast microscope on the addition of homologous antibody. The cell-wall structure, as revealed in the specific cell-wall reaction, is very similar to that observed in stained preparations. A method is described for staining the bacterial cell wall with Alcian blue without preliminary treatment with a mordant.

Tomcsik & Guex-Holzer (1952, 1954*a*) showed that when the cell wall of '*Bacillus* M' was separated from the cytoplasm by the action of trypsin or lysozyme, it could be made visible to phase-contrast microscopy by reaction with an antibody specific for a bacillary polysaccharide. This phenomenon, designated the specific cell-wall reaction, was also observed in *B. anthracis* but only after prior treatment with trypsin (Tomcsik, 1954). Many bacteria, however, cannot be digested with lysozyme or trypsin and one purpose of the present investigation was, therefore, to determine whether this reaction occurred in cell walls obtained by mechanical disintegration (Salton & Horne, 1951).

The standard cytological methods for the demonstration of the bacterial cell wall entail the use of a mordant followed by the application of a basic dye (Robinow, 1946; Hale, 1953; Bisset & Hale, 1953). Recently, Alcian blue has been recommended as a stain for bacterial capsules (Novelli, 1953). We have found, however, that Alcian blue will stain the cell walls of many bacteria without the use of a mordant. In this study we have used these staining methods in parallel with the specific cell-wall reaction to compare the cell walls of intact bacteria with those obtained after enzymic or mechanical disruption.

METHODS

Organisms. The following strains of bacteria were selected for examination, partly on account of their notably multicellular morphology: *Bacillus* M described by Tomcsik & Guex-Holzer (1951, 1954*a*, *b*); *Bacillus anthracis* strain Vollum, NCTC 7200; *B. megaterium*, strain I from Dr J. P. Aubert, Institut Pasteur, and strain II from Dr S. Raffel, Stanford University, California, U.S.A.; *Micrococcus* sp., strains A and B; *Caryophanon latum* from Dr K. A. Bisset, University of Birmingham.

Culture media. Caryophanon latum was grown on agar containing 0·5 % (w/v) Difco peptone, 0·5 % yeast extract and 0·01 % sodium acetate. The other bacteria were grown on Gladstone & Fildes CCY agar or broth, without the addition of glucose. The capsulated cells of *Bacillus* M were obtained from cultures grown on the agar at 28° and the non-capsulated form from the broth which was aerated for 10–18 hr. at 28°.

Antibodies. The polysaccharide antibody against *Bacillus* M and the D-glutamic acid polypeptide antibody were those described in a previous work (Tomcsik & Guex-Holzer, 1954*a*). The homologous antibodies against *B. megaterium*, *B. cereus*, *Micrococcus* spp. and *Caryophanon latum* were produced by the intravenous inoculation of rabbits with 10 doses of living suspensions at 3–4-day intervals. Heat-killed suspensions were used in the case of *B. anthracis*. The animals were bled 8 days after the last injection.

Alcian blue. Obtained from Imperial Chemical Industries Ltd., Manchester.

Mechanical disintegration of the cells. Isolated cell walls were obtained by a method similar to that used by Salton & Horne (1951). One volume of smooth beads of average diameter 0·2 mm. was added to three volumes of a thick bacterial suspension and the whole shaken for 10–120 min. in a Mickle disintegrator until 90 % of the cells were free from protoplasts when examined microscopically. The amplitude of vibration was kept constant for each species. The isolated cell walls were then washed with physiological saline.

Trypsin digestion. Two commercial preparations of trypsin were used: Trypsin I (Merck, Darmstadt) and trypsin II (Siegfried, Zofingen). Ten mg. trypsin were suspended in 1·0 ml. phosphate buffer (pH 8·0). Three ml. of a similarly buffered bacterial suspension, the turbidity of which corresponded to tube 4 of the McFarland $BaSO_4$ series (Stitt, Clough & Clough, 1939), was added together with 1 ml. chloroform. The suspension was incubated at 37° for 10–18 hr., the degree of digestion being determined by phase-contrast microscopy.

Lysozyme digestion was carried out by the method of Tomcsik & Guex-Holzer (1952).

Phase-contrast preparations for the specific cell-wall reaction were made as described by Tomcsik & Guex-Holzer (1954*a*). The currents set up in the wet mount by the addition of the serum removed many of the cell walls from the cover glass and made difficult the preparation of serial photomicrographs of the same cells before and after the addition of antiserum (Pl. 2, figs. 11, 12).

Staining methods. The cells were fixed, when not otherwise stated, for 2–3 min. in the vapour from 1·0 % osmic acid. The cell walls were stained by the tannic acid violet method (Robinow, 1946), by the phosphomolybdic acid + methyl green method (Hale, 1953), and by the following modification of the technique described by Novelli (1953): smears prepared on coverslips were either allowed to dry in the air or were fixed with osmic acid vapour. An ethanolic solution of the stain (Alcian blue 1 g. in 100 ml. 95 % ethanol) was diluted 1/10 with distilled water and the coverslips immersed in it for 5–10 min., except with *Caryophanon latum*, the cell wall of which required 30–45 min. Preparations were then washed and mounted in water.

RESULTS

The specific cell-wall reaction was elicited after enzymic or mechanical disintegration. Treatment of *Bacillus* M with lysozyme separated the cell wall from the protoplast and consequently gave a very clear-cut demonstration of the specific cell-wall reaction on the addition of polysaccharide antibody (Tomcsik & Guex-Holzer, 1952, 1954*a*, *b*). With the exception of *B. megaterium*, however, the bacteria examined were resistant to the action of lysozyme and this method could not be applied to them. Similarly, no digestion of the cells of *B. cereus* and *Micrococcus* spp. was observed after treatment with trypsin, and only variable results were obtained with *Caryophanon latum*. There was, however, a marked destruction of the cytoplasm of *Bacillus* M, *B. megaterium* and *B. anthracis* with trypsin, and a characteristic morphological change was observed by phase contrast which was quite distinct from that produced by crystalline ribonuclease (Mann Research Laboratories). The cell walls and the remainder of the cell contents were indistinct (Pl. 2, fig. 10), although the contents could still be stained clearly by carbol fuchsin (Pl. 2, fig. 9). Homologous antibody, however, produced a strong specific cell-wall reaction (Pl. 2, figs. 10–12).

In contrast to their varied susceptibility to enzymes, the cell walls of all the bacteria examined were consistently separable by shaking the cells in a Mickle disintegrator, although the time required to obtain a 90 % yield varied considerably with the strain. *Micrococcus* A, for example (Pl. 3, fig. 19*c*) a capsulated strain, required 120 min., *Micrococcus* B 30 min. The cell wall broke more often in *Bacillus cereus* and in *B. anthracis* than in *Bacillus* M.

The cell walls of *Bacillus* M obtained by this method differed from those of the other bacteria in that they were barely visible in the phase-contrast microscope until after the addition of antibody. Those of *B. anthracis* and *B. cereus*, however, were distinctly seen, although their visibility was enhanced by the addition of antibody (Pl. 2, figs. 13–16). The cell walls of *Caryophanon latum* and particularly those of the *Micrococcus* spp. were so clearly visible after mechanical disintegration that no appreciable thickening could be observed after the addition of homologous antiserum (Pl. 3, figs. 18, 21 and 22), although it contained enough antibody to agglutinate intact cells in dilutions of 1/160 and 1/1024, respectively. Large, spherical cytoplasmic masses were occasionally observed in cell-wall preparations of *C. latum* after the addition of serum, suggesting that a weakening or lesion of the cross-walls had been followed by the fusion of two or three intact protoplasts (Pl. 3, figs. 22, 23). Swelling of material that revealed septation in the form of a Maltese cross (Pl. 3, fig. 19) was observed in the cocci about 1 hr. after the addition of the serum. There was very little cross-agglutination between *Micrococcus* A and B, and the cell-wall reaction was specific.

Cell-wall staining. The cell walls and transverse septa of all the bacteria examined could be stained with Alcian blue without preliminary mordanting. The staining was, however, usually very weak (Pl. 1, figs. 6, 8; Pl. 3, fig. 17) and lacked the distinctness and brilliance obtained by standard methods

(Pl. 1, figs. 5, 7). The very distinct cell-wall structure of *Caryophanon latum* seen in Pl. 3, fig. 20, was obtained by prolonged staining (40 min.) and the use of an orange filter for photography. In cell walls separated from the intact protoplast by mild lysozyme digestion, both the cell wall and the membrane were apparently stained by Alcian blue (Pl. 1, fig. 8) and by Hale's method (Pl. 1, fig. 7).

The cell-wall structure revealed by the specific cell-wall reaction was very similar to that elicited by staining. The capsular transverse septa of *Bacillus* M, however, could be demonstrated only with polysaccharide antisera and could not be stained by any of the methods used in this study (Pl. 1, figs. 1, 2). An indistinct capsule could be demonstrated with Alcian blue (Pl. 1, fig. 4), but it resembled that obtained after reaction with polypeptide antibody (Pl. 1, fig. 3).

DISCUSSION

Our observations show that the specific cell-wall reaction originally applied to *Bacillus* M can be elicited in a variety of bacteria including *Caryophanon latum*. We consider that this reaction may be of value, therefore, in the investigation of the mode of action of immunochemically defined antibodies on bacteria as well as in the location of serologically active substances within the cell.

The specific cell-wall reaction demands a preliminary treatment of the bacteria in order to separate the cell wall from the cytoplasm. Previous observations (Tomcsik & Guex-Holzer, 1954*a*), as well as those described here, are contrary to the general concept based on the work of Kantorowicz (1909) and Salton (1953) that neither living nor heat-killed cells of Gram-positive bacteria can be digested to any extent with trypsin. The present work suggests, however, that a re-investigation of this question is necessary since the cell contents of three of the five Gram-positive bacteria we studied, *Bacillus* M, *B. megaterium* and *B. anthracis*, were markedly digested by trypsin, whereas *B. cereus* and species of *Micrococcus* were resistant to this enzyme. The effect of trypsin on these species of *Bacillus* cannot be regarded as of universal application until a more representative number of strains has been examined. Experimental evidence (Tomcsik & Guex-Holzer, 1954*c*) suggests that there is a thermolabile protein on the surface of *Bacillus* M which is readily destroyed by trypsin, and consequently its removal might afford direct access of the trypsin to the protoplast.

The separation of the cell walls by mechanical disintegration by the method of Salton & Horne (1951) does not alter essentially the morphological appearance of the cell-wall structure. The cross-walls of the *Bacillus* spp. are almost unaltered, although chain breakage and fissures in the cell walls can often be seen by the phase-contrast microscope. A similar observation was made by Mudd, Polevitzky, Anderson & Chambers (1941) in their electron micrograph studies of bacilli treated in a sonic oscillator, although comparison of their photomicrographs with ours suggests that by their method there was considerably less destruction of the protoplasts. Murray & Robinow (1952) obtained similar results with cells of *B. cereus* that had been accidentally crushed with cover-glass forceps. By mechanical disintegration, the two right-angled cross-walls of *Micrococcus* spp. discovered by Bisset (1948, 1954) in stained preparations were readily demonstrated by phase contrast without the addition of antibodies.

The appearance of the cell walls after staining with Alcian blue was essentially similar to that obtained by the standard methods which involve mordanting. Eisenberg (1910) assumed that mordants such as tannic acid and mercuric chloride react with the surface proteins of the bacterial cytoplasm (Endoplasma) and that subsequent staining is restricted to the cell wall (Membran) and the cytoplasmic membrane (Rindenschicht). Using a new method, Robinow & Murray (1953) have demonstrated that both the cell wall and membrane are stained after treatment with mordants. Bisset (1953) is of the opinion that the cell membrane as such is probably destroyed during mordanting and that the basophilic elements are precipitated upon the wall. Both views may well be correct

It can be seen from the two photomicrographs (Pl. 1, figs. 7, 8) that when the cell wall and cytoplasmic membrane of *Bacillus* M are separated by mild lysozyme digestion, both structures are stained by Alcian blue. The staining of the cell wall may represent a microchemical polysaccharide reaction. McKinney (1953) has shown that of a number of organic substances, only carbohydrates retained the dye when it was applied in a 95% ethanolic solution. The recent report of Grula & Hartsell (1954) that Alcian blue stains the cell wall of *Micrococcus lysodeikticus*, is also consistent with this view. The staining of the surface of the cytoplasm after the separation of the protoplast may be due, however, to the protein precipitating action of the dye.

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EXPLANATION OF PLATES

All plates at × 2000. ph.c. = phase-contrast.

Plate 1

Fig. 1. Capsulated *Bacillus* M, ph.c., no antibody.
Fig. 2. Capsulated *Bacillus* M, ph.c., polysaccharide antibody.
Fig. 3. Capsulated *Bacillus* M, ph.c., first polysaccharide, afterwards polypeptide antibody (the same microscopic field as in Fig. 2).
Fig. 4. Capsulated *Bacillus* M, Alcian blue.
Fig. 5. Non-capsulated *Bacillus* M, tannic-acid-violet.
Fig. 6. Non-capsulated *Bacillus* M, Alcian blue.
Fig. 7. Non-capsulated *Bacillus* M, treated with 1:400 egg-white at room temperature, 25 min. Hale's cell wall stain.
Fig. 8. Same as Fig. 7. Alcian blue.

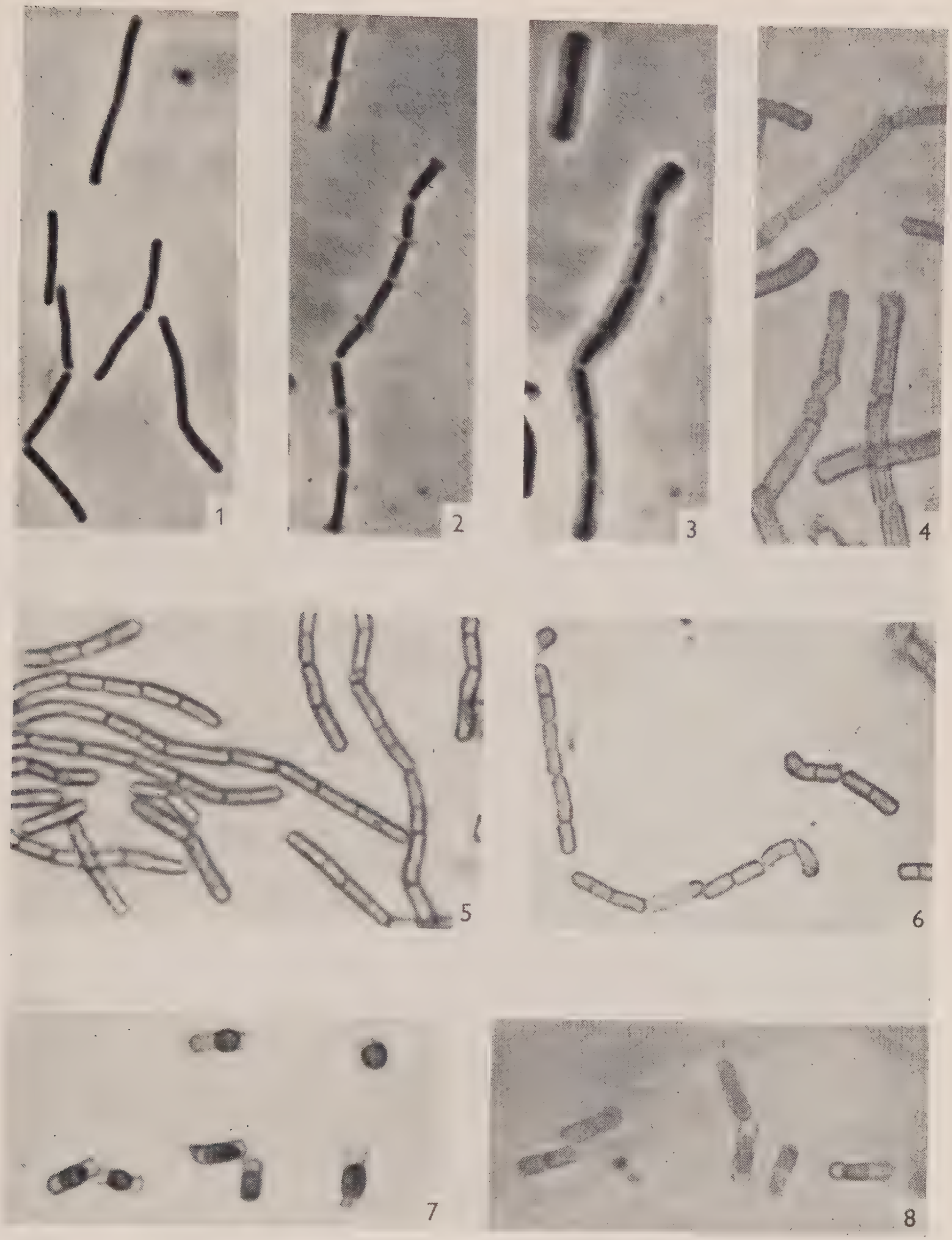
Plate 2

Fig. 9. Non-capsulated *Bacillus* M, digested with trypsin. Alcian blue, carbol fuchsin.
Fig. 10. Non-capsulated *Bacillus* M, digested with trypsin, ph.c. Polysaccharide antibody.
Fig. 11. Non-capsulated *Bacillus* M, digested with trypsin, ph.c. No antibody.
Fig. 12. The same microscopic field as in fig. 11, ph.c. Polysaccharide antibody.
Fig. 13. *B. cereus*, Mickle-vibrator 15 min. ph.c. No serum.
Fig. 14. *B. cereus*, Mickle-vibrator 15 min. ph.c. Homologous serum.
Fig. 15. *B. anthracis*, Mickle-vibrator 15 min. ph.c. No antibody.
Fig. 16. *B. anthracis*, Mickle-vibrator 15 min. ph.c. Polysaccharide antibody.

Plate 3

Fig. 17. Coccus A, Alcian blue.
Fig. 18. Coccus A, Mickle-vibrator 2 hr. 20 min., ph.c. No serum.
Fig. 19. Coccus A, Mickle-vibrator 2 hr. 20 min., ph.c. Homologous serum.
Fig. 20. *Caryophanon latum*, Alcian blue, orange filter.
Fig. 21. *C. latum*, Mickle-vibrator 20 min., ph.c. Homologous serum.
Fig. 22. Same as Fig. 21, ph.c., showing one large spherical protoplast.
Fig. 23. *C. latum* with homologous serum, 4 hr. in wet chamber. An unusual form with spherical elements.

(*Received* 19 *January* 1955)



J. Tomcsik and J. B. Grace -Cell wall reactions. Plate 1

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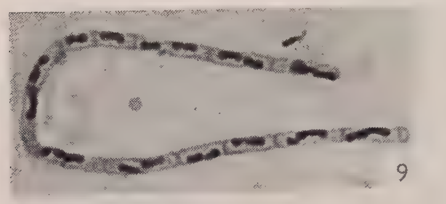
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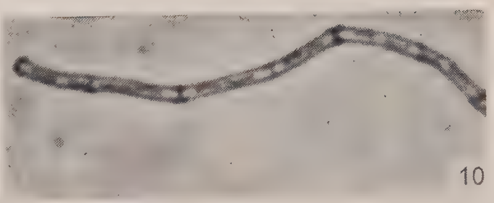
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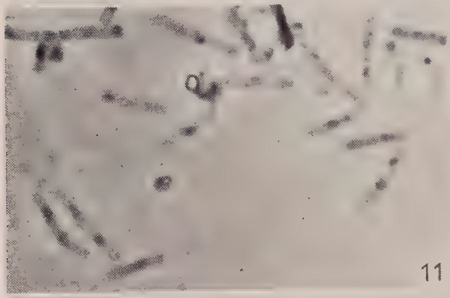
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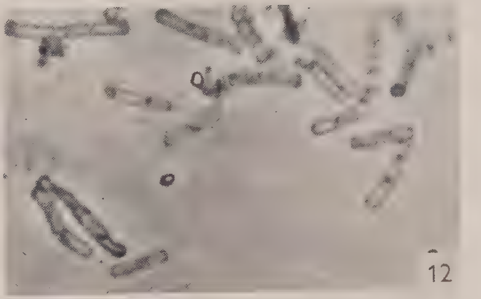
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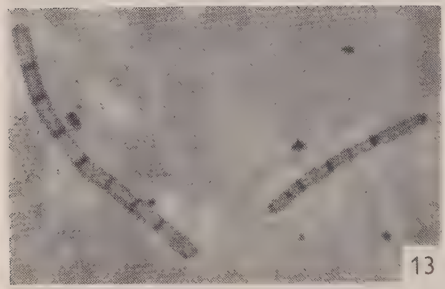
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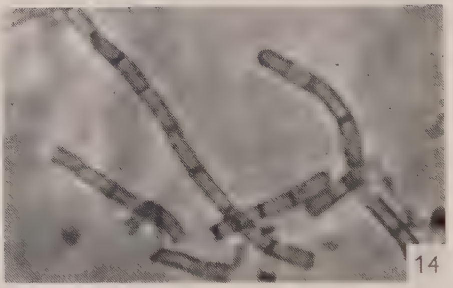
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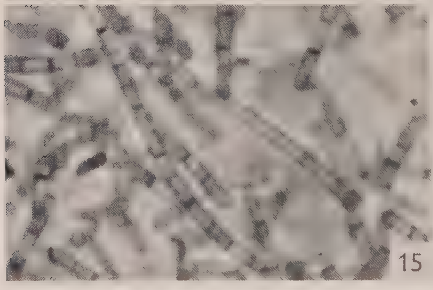
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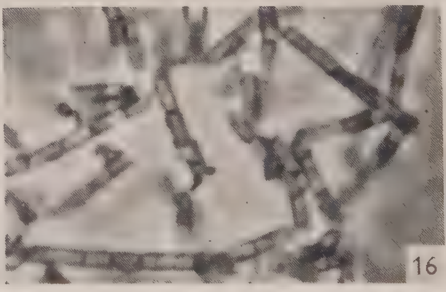
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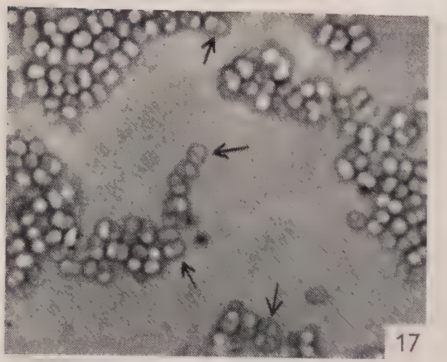
J. TOMCSIK AND J. B. GRACE—CELL WALL REACTIONS. PLATE 2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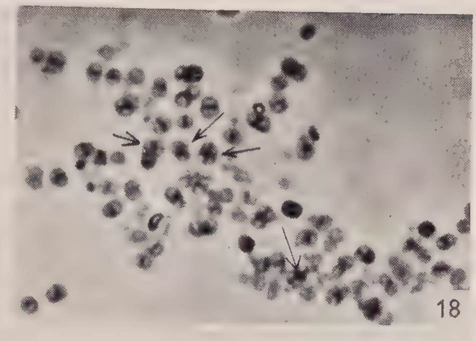
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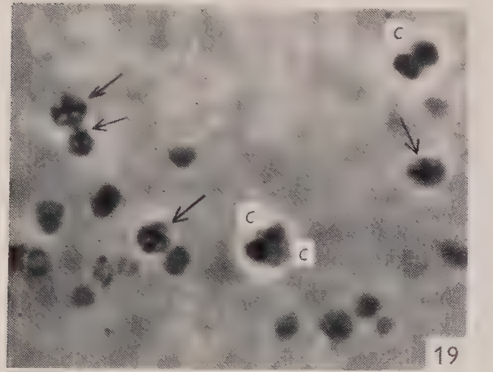

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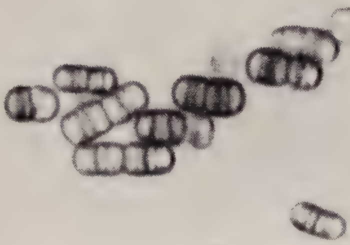
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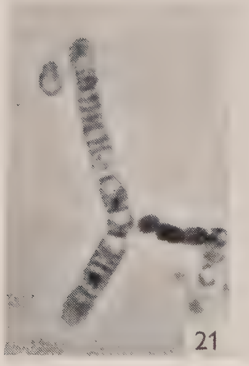
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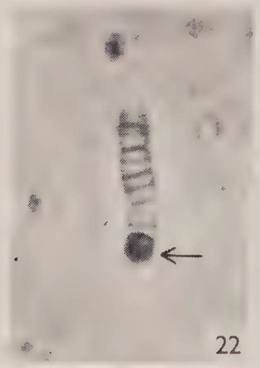
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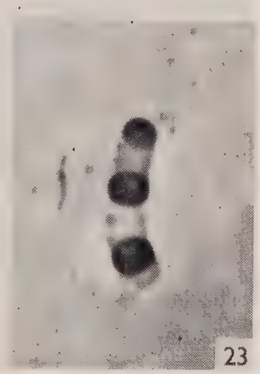
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J. Tomcsik and J. B. Grace—Cell wall reactions. Plate 3

SPENCER, R. (1955). *J. gen. Microbiol.* **13**, 111–118.

The Taxonomy of certain Luminous Bacteria

BY R. SPENCER*

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SUMMARY: A named strain of each of the species *Photobacterium phosphoreum*, *P. fischeri*, *P. albensis*, *P. pierantonii*, *P. splendidum*, *P. sepia* and *P. harveyi*, together with a recently isolated strain of each of the first two named species, were studied in an attempt to elucidate their correct generic classification. On the basis of morphology, sensitivity to certain antibiotics and a vibriostatic substance, and method of dissimilation of carbohydrates, it was concluded that *P. sepia* and *P. harveyi* should be classified in the genus *Aeromonas*, and the remainder of the species in the genus *Vibrio*.

Although there have been many studies of the mechanism of light production by luminous bacteria (Harvey *et al.* 1948), comparatively few have been devoted to the taxonomy of this group. The work done has been complicated by the fact that these organisms belong to the large group of Gram-negative, non-pathogenic, non-pigmented rods whose differentiation has always been a difficulty. The Dutch workers, following the lead of Beijerinck (1889), classify all luminous bacteria in the genus *Photobacterium* regardless of their differences. This practice has much to commend it from the determinative viewpoint, but it may result in bacteria which belong to different morphological groups being classified together. In *Bergey's Manual* (1948) luminous bacteria are placed in the genera *Pseudomonas*, *Vibrio* and *Bacterium*. As the latter scheme bases the differentiation between *Pseudomonas* and *Vibrio* upon presence or absence of curvature in the bacterial cell, a very variable characteristic, and as the genus *Bacterium* in the *Manual* is an acknowledged collection of unrelated groups, it does not clarify matters. A recent paper by Breed & Lessel (1954) reviewed the literature on the nomenclature of luminous bacteria, but presented no new facts concerning their taxonomy. In recent years, new criteria have been evolved for the differentiation of certain groups of Gram-negative rods, notably the significance of the type of carbohydrate dissimilation (Hugh & Leifson, 1953), and the inhibition by various antibiotics (Shewan, Hodgkiss & Liston, 1954). This paper records the results of applying these and other criteria to a number of strains of luminous bacteria, as part of a general study of the bacterial flora of marine fish, of which luminous bacteria form a not inconsiderable part (Stewart, 1932; Aschehoug & Vesterhus, 1943; Thjøtta & Sømme, 1943; Liston, 1955).

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METHODS

One strain of each of the species *Photobacterium phosphoreum*, *P. sepia*, *P. albensis*, *P. pierantonii*, *P. harveyi* and *P. splendidum* were obtained from Professor A. J. Kluyver of Delft. One strain of *P. fischeri* was obtained from Professor J. Cruickshank of Aberdeen University. Strain 2 was isolated from the gut contents of skate and is representative of over fifty almost identical strains obtained from North Sea fish and classified as *P. phosphoreum*. Strain 64 was obtained from the gut contents of mackerel and is classified as *P. fischeri*.

The solid media used consisted of tap-water Lemco agar (Lemco, 10 g.; peptone (Oxoid), 10 g.; NaCl, 5 g., tap water, 1000 ml.; pH 7·6) and sea-water Lemco agar (Lemco, 10 g.; peptone, 10 g.; tap water, 250 ml.; aged sea water, (ZoBell, 1946), 750 ml.; pH 7·6). Tap-water peptone water (peptone, 10 g.; NaCl, 0·5 g.; tap water, 1000 ml.; pH 7·6) or sea-water peptone water (peptone, 10 g.; tap water, 250 ml.; aged sea water, 750 ml.; pH 7·6) were used for liquid media. For the biochemical tests tap-water media alone were used as it was found that these gave good growth in all cases.

For the antibiotic sensitivity tests, tap-water Lemco agar was used as sea water inhibited the action of terramycin to some extent. Tablets containing the indicated amounts of antibiotic were used for penicillin, chloramphenicol, streptomycin and terramycin, and the cup assay method with a saturated aqueous solution for the vibriostatic substance O/129 (2-4-diamino, 6-7-di-isopropylpteridine; Shewan *et al.* 1954). The agar plates were surface-poured with a young culture and dried at room temperature for some hours before the antibiotics were applied.

In the determination of the type of dissimilation of carbohydrates, Hugh & Leifson's (1953) medium was used, but with 1·5 % NaCl instead of 0·5 %. Glucose was the only carbohydrate used as Hugh & Leifson found that organisms which oxidize glucose, but do not ferment it, do not ferment other carbohydrates.

Unless otherwise stated, the temperature of incubation was always at 20°.

Observations on morphology were made on cultures growing on tap-water and sea-water Lemco agar, and in tap-water and sea-water peptone water, at various temperatures, using stained preparations, and also wet preparations by phase-contrast microscopy. Determinations of motility were made on cultures growing in tap-water and in sea-water peptone water, again at various temperatures.

The methods used for the biochemical tests are given in the appropriate column in Table 1. Sugar fermentation and gelatin liquefaction tests were examined up to 28 days of incubation, and the following sugars were used: glucose, lactose, sucrose, mannitol, maltose, starch, glycerol, inositol, rhamnose, xylose, inulin, galactose, glycogen, raffinose, arabinose, sorbitol. The Voges-Proskauer, methyl red, indole production, and nitrate reduction tests were examined after 7 days, and the trimethylamine-oxide reduction test after 9 days of incubation. The trimethylamine-oxide reduction test was used as an index of possible importance in fish spoilage. Growth temperatures were determined after 72 hr. of incubation in peptone water, with tap water for *Photobacterium albensis* and sea water for the other strains.

RESULTS

The cultural and biochemical characteristics of the strains of luminous bacteria were first determined (Table 1) and compared with previously published characteristics, notably those in *Bergey's Manual* (1948), in Warren's (1945) study of several species of luminous bacteria, in the original description by Johnson & Shunk (1936) of *Photobacterium harveyi*, and a full description of *P. fischeri*. This was necessary to ensure the validity of the named strains and the correct identification of the two unnamed strains.

There appeared to be several differences between the characteristics found in the present study and those previously described. Besides slight anomalies in the production of acid in various sugars, particularly the inability of strain 2, *Photobacterium harveyi* and *P. phosphoreum* to produce acid in sucrose, the main discrepancies were the inability of *P. fischeri* and *P. pierantonii* to liquefy gelatin, of *P. splendidum* to grow at 30 and 37°, and the non-motility of *P. albensis* and *P. pierantonii*. These discrepancies will be discussed later.

The antibiotic sensitivity patterns are shown in Table 2. According to the classification of Shewan *et al.* (1954), *Photobacterium albensis* should be classified as a member of the Achromobacteriaceae because it shows sensitivity to penicillin and to streptomycin. However, the previously described single polar flagellation of this organism and its morphology, vibrio- or spirillum-like, would preclude this classification. Also, as the degree of inhibition is very slight, much less than is generally obtained with tested members of the genus *Achromobacter*, it is considered that *Photobacterium albensis*, like the others, should be placed in the family Pseudomonadaceae. Within this family the species fall into two groups, those sensitive to the vibriostatic compound O/129, and those (*P. sepia* and *P. haveyi*) which are insensitive.

The method of dissimilation of glucose by the various species was in every case fermentative, thus confirming the results of Doudoroff (1942).

The criterion generally used for differentiating the genera *Pseudomonas* and *Vibrio* is that of curvature of the cell, which is present in vibrios and absent from pseudomonads. Consequently, the morphology of the various species was investigated in liquid and on solid media. It immediately became apparent with *Photobacterium phosphoreum* and strain 2 that there were marked discrepancies between previously published descriptions and those now observed. Beijerinck (1889), Stewart (1932), Warren (1945) and *Bergey's Manual* (1948) all emphasized the coccal or coccobacillary morphology of *P. phosphoreum*, but unfortunately none gave the medium used, although Beijerinck did record that they 'prendre dans certaines conditions la forme de spirilles et de vibrions'. Liston (personal communication) has pointed out that although *P. phosphoreum* is coccal or coccobacillary when grown in both tap-water and sea-water peptone water, on tap-water Lemco agar vibrio- and spirillum-like

Table 1. *Characteristics of luminous bacteria*

	Organism								
	Strain 2	*P. fischeri*	*P. sepia*	*P. albensis*	*P. pierantonii*	*P. harveyi*	*P. splendidum*	*P. phosphoreum*	Strain 64
Degree of curvature of the cell	Marked and frequent	Marked and frequent	Occasional and slight	Marked and frequent	Occasional and slight	Very occasional and slight	Marked and frequent	Marked and frequent	Marked and frequent
Motility	+	+	+	−	−	+	+	−	+
Flagella (Casare-Gill stain)	Single polar	Single polar	Single polar	−	−	Single polar	Single polar	−	Single polar
Growth at 0°	+	−	−	−	−	−	−	+	−
20°	+	+	+	+	+	+	+	+	+
30°	−	+	+	+	+	+	−	−	−
37°	−	−	+	+	+	+	−	−	−
Growth at 20° anaerobically	+	+	+	+	+	+	+	+	+
Preference for sea-water media	+	+	+	−	+	+	+	+	+
Gelatin liquefaction	−	−	+	+	−	+	+	−	+
Nitrate reduction (Griess Ilosva, Topley & Wilson, 1946)	+	+	+	+	+	+	+	+	+
Trimethylamine oxide reduction (Wood & Baird, 1943)	+	+	+	+	+	+	+	+	+
Methyl red	−	−	−	−	−	−	−	−	−
Voges-Proskauer (Barritt, M. M., 1936)	+	−	−	+	−	−	−	+	−
Indole (Böhme, A., 1906)	−	−	+	+	−	+	+	−	−
Gas production in sugars	+	−	−	−	−	−	−	+	−
Acid in the following sugars	Glucose Galactose Lactose Maltose	Glucose Glycerol Mannitol Galactose Maltose Glycogen Starch	Glucose Sucrose Mannitol Maltose Starch Galactose Glycogen	Glucose Sucrose Maltose Starch Galactose Glycogen Arabinose	Glucose Mannitol Maltose Starch Glycerol Galactose Glycogen	Glucose Mannitol Maltose Starch Galactose Glycogen	Glucose Lactose Mannitol Maltose Starch Glycerol Galactose Glycogen	Glucose Maltose Galactose	Glucose Sucrose Mannitol Maltose Galactose

+ = positive; − = negative.

forms are produced. This observation has been confirmed and extended to sea-water Lemco agar (Pl. 1, figs. 1–3). The presence or absence of curvature in the cells of the other species is recorded in Table 1, and typical appearances are shown in Pl. 1, figs. 4–6.

Table 2. *Sensitivity of luminous bacteria to antibiotics*

Organism	Penicillin, 2·5 i.u.†	Chloramphenicol, 100 μg.†	Streptomycin, 80 μg.†	Terramycin, 10 μg.†	Vibriostatic compound O/129*
Strain 2	−	+	+	+	+
P. fischeri	−	+	+	+	+
P. sepia	−	+	+	+	−
P. albensis	∓	+	+	+	+
P. pierantonii	−	+	+	+	+
P. harveyi	−	+	+	+	−
P. splendidum	−	+	+	+	+
P. phosphoreum	−	+	+	+	+
Strain 64	−	+	+	+	+

+, sensitive; −, insensitive; ∓, slight degree of sensitivity.
* 2–4-diamino, 6–7-di-isopropyl pteridine.
† See p. 112.

DISCUSSION

Certain discrepancies have been noted between characteristics of the luminous bacteria examined, and previous descriptions in the literature. Both *Photobacterium fischeri* and *P. pierantonii* were found not to liquefy gelatin, although the recently isolated strain closely resembling *P. fischeri* does liquefy gelatin. *P. fischeri* was recorded by Johnson & Shunk (1936) and by Warren (1945) as liquefying gelatin only weakly, *Bergey's Manual* (1948) recorded both *Pseudomonas pierantonii* and *Vibrio pierantonii* as not liquefying gelatin, whilst Warren (1945) recorded *Photobacterium pierantonii* as liquefying gelatin. Consequently, the discrepancies found are not thought important.

It is known that the ability to liquefy gelatin is frequently lost on continued subculture on artificial media, as is the property of motility which also appears to have been lost by the strains of *Photobacterium pierantonii* and *P. albensis* studied. The lowering of the maximum growth temperature of *P. splendidum* below 30° may also come into the category of properties lost due to continued subculture in artificial media, particularly as the other characteristics of this species correspond to those previously published. Johnson, Zworykin & Warren (1943) obtained electron micrographs of *P. fischeri* showing lophotrichous flagellation. In the present study this organism was found to possess a single polar flagellum. The difference may merely be due to the different methods of examination.

It thus appears that the strains of luminous bacteria studied are typical members of the named species. Also, although this is not the main purpose of the present work, it appears that the strains of the named species studied have sufficiently distinct characteristics to be regarded as separate species, with the possible exception of *Photobacterium sepia* and *P. harveyi*. Previous work, notably by Warren (1945), together with the present work, has shown these species to be very similar.

A Gram-negative heterotrophic rod which is motile by one or more polar flagella, is easily classified in the family Pseudomonadaceae. If, however, the rod is non-motile, difficulties are immediately found, but by determining the antibiotic sensitivity pattern of the organisms these difficulties can generally be resolved. By this criterion, the non-motile luminous bacteria studied fall into the Pseudomonadaceae, and the classification of the motile species in this family on the basis of their polar flagellation is confirmed. Within this family the strains can only fall into the genera *Pseudomonas* or *Vibrio*, or possibly the genus *Aeromonas* (Kluyver & van Niel, 1936). On the basis of curvature of the cell, it can be seen (Table 1) that some of the strains fall into the genus *Vibrio*, whilst the remainder, on this criterion alone, should be placed in the genus *Pseudomonas*. All those species showing marked curvature of the cell are also sensitive to the vibriostatic substance, and, as they evince a fermentative dissimilation of glucose, there seems no reason why they should not be placed in the genus *Vibrio*. These species are *Photobacterium fischeri* and strain 64, *P. phosphoreum*, and strain 2, *P. albensis* and *P. splendidum*.

Photobacterium pierantonii shows only slight curvature, but is sensitive to the vibriostatic substance. Many strains of pigmented and non-pigmented pseudomonads, including some *Aeromonas* species, have been examined, and have all been found to be insensitive to the vibriostatic substance (Spencer & Hodgkiss, unpublished). Consequently, it is considered that despite its lack of marked curvature, *P. pierantonii* should be classified as a vibrio.

The two remaining species, *Photobacterium sepia* and *P. harveyi*, due to their lack of curvature and resistance to the vibriostatic compound, cannot be placed in the genus *Vibrio* although they do dissimilate glucose fermentatively. Kluyver & van Niel (1936) suggested that pseudomonads possessing a fermentative metabolism should be separated from those which possess an oxidative metabolism similar to that of *Pseudomonas aeruginosa*, the type species. For the group of fermentative pseudomonads they proposed the generic name *Aeromonas*, a suggestion which has been repeated by several subsequent workers. *Photobacterium sepia* and *P. harveyi* should fall into this genus, except that as originally defined by Kluyver & van Niel, members of the genus should ferment carbohydrates with the production of carbon dioxide and hydrogen. Strains of non-luminous *Aeromonas* studied by the author have this property, but neither *Photobacterium sepia* nor *P. harveyi* produces gas from carbohydrates, or does so only in very small amounts (Doudoroff, 1942). Despite this, it is considered that these two species should be placed in the genus *Aeromonas*, the definition of which should be amended so as not to exclude anaerogenic fermentative pseudomonads. This would be preferable to creating a new genus on the present insufficient knowledge of the metabolism of pseudomonads.

As luminous bacteria have been shown to fall into two distinct genera, indeed, into two distinct tribes in the generally accepted taxonomic scheme, i.e. Pseudomonadeae and Spirillae, it is thought unwise to group them together in a common genus *Photobacterium*. The suggested generic classification of the luminous bacteria is that the species *phosphoreum*, *albensis*, *pierantonii*, *splendidum* and *fischeri* be placed in the genus *Vibrio*, whilst *sepia* and *harveyi* be placed in the genus *Aeromonas*. This classification is substantially the same as that proposed by Breed & Lessel (1954) who, however, proposed that luminous bacteria of coccal or coccobacillary form should be placed in a separate genus, *Photobacterium*. As *P. phosphoreum* has been shown to be a curved rod on solid media, and, moreover, sensitive to the vibriostatic agent, this separation is considered unnecessary.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

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EXPLANATION OF PLATE

Fig. 1. Strain 2, tap-water peptone water culture, 18 hr. at 20°, showing coccal and coccobacillary forms. × 768.

Fig. 2. Strain 2, sea-water Lemco agar culture, 24 hr. at 20°, showing curved rods. × 768.

Fig. 3. *P. phosphoreum*, sea-water Lemco agar culture, 24 hr., at 20°, showing markedly curved forms. × 960.

Fig. 4. *P. splendidum*, sea-water peptone water culture, 72 hr. at 20°, showing curved rods. × 768.

Fig. 5. *P. albensis*, tap-water peptone water, 18 hr. at 20°, showing markedly curved forms. × 768.

Fig. 6. *P. sepia*, sea-water Lemco agar, 48 hr. at 20°, showing typical straight rods. × 768.

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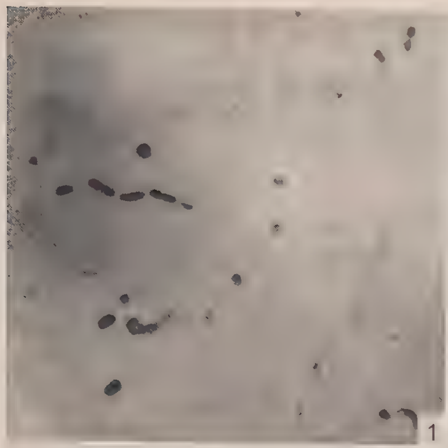

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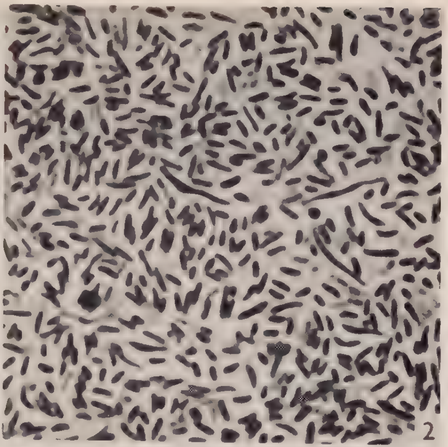

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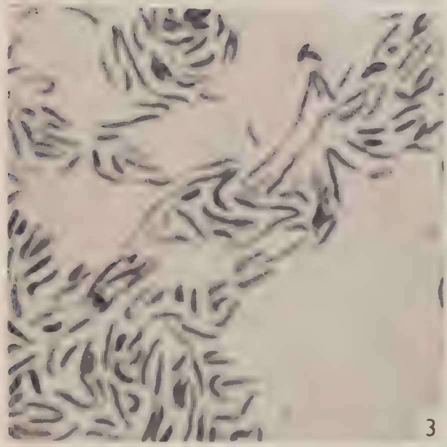

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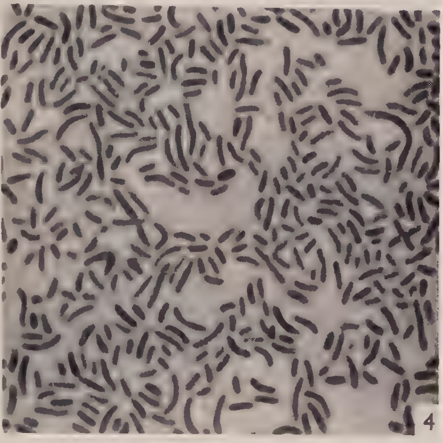

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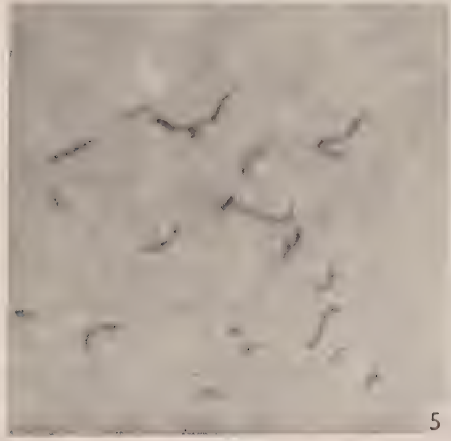

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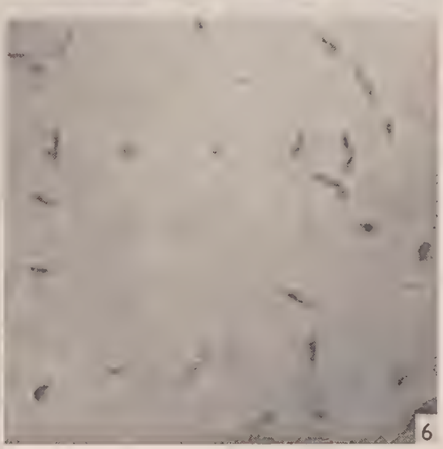

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R. Spencer—Taxonomy of luminous bacteria. Plate 1

(*Facing p.* 118)

Erikson, D. (1955). *J. gen. Microbiol.* **13**, 119–126

Changes in Refractility and Permeability accompanying Germination of Heat-resistant Spores of *Micromonospora vulgaris*

By DAGNY ERIKSON

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SUMMARY: Progressive stages in the germination at 60° of the highly refractile spores of *Micromonospora vulgaris* were observed by means of phase-contrast microscopy. The development of individual spores was followed in slide cultures. Mass cultures were also prepared in liquid and solid media from spore suspensions in phosphate buffer which had been heated at 100° for periods of 1–20 min. and 30 min.–4 hr. The liquid cultures derived from heated and unheated spores were sampled at frequent intervals and examined in wet preparations containing dilute toluidine blue. Throughout, loss of refractility preceded germination, which occurred in 1–3 hr. Germinating spores appeared as dense opaque bodies by phase contrast and were then capable of absorbing the dyestuff to which non-germinating spores were impermeable.

Heat activation at 100° for 1 min. enhanced the initial germination rate within the first 3 hr. period. No differences were observed between heated and unheated spores during the processes of germination. Indications of an eccentric 'nucleus' were occasionally found in phase-contrast examination of very lightly stained fixed spores from material heated for short periods, or in electron micrographs of unheated growths. Heating at 100° for the shorter periods (1–10 min.) resulted in a higher proportion of the spores becoming darkened than was the case with the longer exposures. Artificial darkening of the spores in the manner successfully used for eubacterial spores was not accomplished. Sectored colonies—the sectors showing loss of aerial mycelium and impaired viability—frequently developed from spores which had been subjected for more than 1 hr. to a temperature of 100°.

Intense refractility is an outstanding property possessed by the spores of organisms of the genus *Micromonospora* in general (Erikson, 1949) and especially by the thermophilic species (see original description by Tsiklinsky, 1899, under the name of *Thermoactinomyces vulgaris*). Impermeability to ordinary bacteriological stains is to a certain extent also a character of the mature spores of *Micromonospora vulgaris*, although as pointed out by Erikson (1952) they show up well with Gray's spore stain. In phase-contrast preparations the spores appear as clear or as dense round bodies according to the age of the specimen and the technique used in preparing it for microscopical examination. This study follows the germination of individual spores without previous heat treatment, and of spores in mass cultures subjected to different types of heat treatment.

METHODS

Slide cultures were prepared by placing a drop of molten CPS agar medium (for details of composition see Erikson, 1952) in a hollow-ground slide, seeding it with a dilute suspension of spores, and sealing with a coverslip. Suitable fields with spores well dispersed were charted, their positions noted by means of the vernier readings of the mechanical stage, and photographed. The complete microscope stage, which was movable, was placed for incubation at 60° in a moist chamber. At definite intervals of time the stage was taken out of the incubator, replaced on the microscope, and the field under examination quickly found and photographed; after which the stage was returned to the incubator with the least possible loss of time.

For the heat treatment tests spores were harvested as follows: the 2-day growth on cellophan over a plate of CPS medium was washed off with distilled water, lightly shaken, filtered through sterile glass wool, centrifuged and resuspended in M/75 phosphate buffer (pH 7·0) according to methods described by Erikson & Webley (1953) and by Webley (1954). Since it was desired to have as many spores as possible under observation simultaneously, and as the proportion of viable spores under these artificial conditions was low, no attempt was made to dilute the suspensions or to obtain approximate counts as was done previously (Erikson, 1952). Each plate yielded *c*. 2 ml. dense suspension which was placed for heating in a screw-capped bottle (5 × 2 cm.). Heating was carried out for varying periods in a steamer at 100°, time being allowed for the internal temperature of the bottles to reach 100°. When the bottles were taken out of the steamer they were immediately plunged into ice. Samples of the heated suspensions were placed in small Durham tubes, centrifuged, the supernatant liquid removed and the spores resuspended in nutrient CPS broth or starch + casein hydrolysate medium (Erikson, 1952). Unheated controls were treated similarly. Incubation was at 60°.

A sample droplet from each heated and control phosphate buffer suspension was used for inoculating the surface of a cellophan circle placed instead of a coverslip over the medium in the well of a hollow-ground slide as above. The series of slide cultures in each experiment were incubated in individual sterile slide boxes containing moistened absorbent cotton-wool and fitted with tight lids. Observations on the development of the heated spores were made at hourly intervals by direct microscopical examination of the growths on the cellophan circles. Representative circles were kept for permanent reference by being mounted like sections on slides (Erikson, 1954). Duplicate Durham tubes containing the liquid cultures were removed from the incubator at the same intervals (2, 3, 4, 18 hr.) as the cellophan slide cultures. Neutral formalin (5 %, v/v) was added to the liquid cultures as fixative.

In this way it was possible to compare simultaneously the development in liquids and on solid surfaces of spores similarly treated before germination. Phase-contrast microscopy was used throughout, employing a Watson 'Bactil' microscope with Watson phase-contrast accessories for the greater part of the study. A Cook, Troughton and Sims microscope with movable stage and phase-contrast substage was used for the examination of the unheated individual spores in slide cultures sealed with cover-glasses.

It was found that those spores which had lost their refractility and which in wet preparations appeared dense by phase-contrast microscopy, were also permeable to a dilute ethanolic solution of toluidine blue (0·005–0·01 %, w/v).

By the simple means of adding one drop of the dye solution to the drop of distilled water in which sample cells from liquid cultures were mounted, a convenient and rapid method was available for checking the validity of the observations made on surface growths. Brilliant preparations could be made by the combined use of phase-contrast microscopy and such a light degree of staining (see Pl. 4, fig. 17).

Detailed tests were made on three different strains (H, B2, D3) of *Micromonospora vulgaris*, exposing the spore suspensions to periods of 1, 5, 10, 15 and 20 min. at 100°. Specimens of all the 7 strains in stock were also subjected to more drastic heating: from 30 min. to 4 hr. at 100°. In these instances very dense suspensions were used, and after being heated the material was washed (centrifuge), and resuspended in liquid medium or diluted and plated in solid medium. Random samplings were made from this material in order to see whether any impairment of the mature growth occurred.

RESULTS

Normal spore germination

The mature spores taken from a 2-day culture (strain B2) when seeded on to the surface of a slide culture and sealed with a cover-slip appeared almost always clear (Pl. 1, fig. 1). With careful examination it was usually possible, however, to find certain individuals whose bodies had darkened naturally. Two such dense spores can be seen within the rectangle drawn round six spores in Pl. 1, fig. 1. It will be noted that of the twenty-five spores dispersed outside the rectangle, no others had darkened before incubation. Before any result was noted or photograph taken, the reality of the phenomenon of darkening was ascertained by focusing upwards and downwards to make certain that the appearance was not due to a difference in planes on the part of the minute spores. After doing this repeatedly the viewer could have no doubt whether or not certain spores within a restricted field were clear or dense (see Pl. 1, fig. 2, an enlargement of the area contained within the rectangle in Pl. 1, fig. 1). It was also possible by taking cover-slip impression preparations of mature growth on plates to examine much larger fields and there determine the great preponderance of clear spores (see Pl. 3, fig. 15).

After incubation for 1 hr. at 60° there was commonly a perceptible darkening of several spores within any given field. Thus in Pl. 1, fig. 4, two more spores had darkened within the rectangular area, while at least another two could be seen outside the rectangle in Pl. 1, fig. 3. A gradual progressive darkening of two or three more spores continued, and after incubation for 3 hr. one of the spores which had been conspicuously refractile before, and which had darkened within the first hour after incubation, had put out a short germ-tube (Pl. 1, fig. 5). It was noteworthy that neither of the two spores within the rectangle which had been dark at the beginning of the experiment showed any further growth. Indeed, by comparison with the germinating spore (Pl. 1, fig. 4; Pl. 2, fig. 6) they had already begun to diminish in volume and appeared to be undergoing the first stages of degeneration.

After incubation for 4 hr. the germinating spore had produced a relatively long filament at an angle from the germ-tube (Pl. 2, fig. 8). The diameter of this filament appeared at this stage uneven along its length, due in part to disorientation as the result of meeting a spore and also to the constriction remaining at the point of origin from the germ-tube. Both the constriction and the pointed bud-like growing point are characteristic features of actinomycetes in general. The boundary lines of the rectangle containing the spores under examination were altered at this stage (Pl. 2, fig. 7) in order to include the full length of the young filament in the photograph. Unfortunately, the further development of this particular spore could not be followed because of the partial drying up of the specimen.

The next stage of early mycelial branching, after incubation for 6 hr., was photographed (Pl. 2, fig. 9) from the growth of another spore outside the rectangle, which had developed simultaneously and part of whose initial filament can be seen in the top right-hand corner of Pl. 2, fig. 7. The germination of this and other spores in the preparation was like that already followed. The 6 hr. growth shown in Pl. 2, fig. 9, developed after incubation for 24 hr. into the typical small mycelium shown in Pl. 2, fig. 10.

Germination of spores subjected to heating for 1–20 *min. at* 100°

The effect of heat activation on *Micromonospora vulgaris* spores was earlier found to be optimum when dilute broth or sucrose suspensions were exposed to a temperature of 85° for 15 min. (Erikson, 1952). In the present work as a result of the use of phosphate buffer suspensions prepared as described by Webley (1954), it was found that in the early stages of germination the spores were responsive to a higher range of temperatures. An examination of the growths obtained on the cellophan slide cultures revealed that the highest *initial* percentage of germinated spores was found 3 hr. after incubation in the set which had been pre-heated at 100° for 1 min., namely: 9 % as compared with 5 % in the case of the unheated control, and 7, 6, 4 and 3 % in the case of the material which had been pre-heated at 100° for 5, 10, 15 and 20 min., respectively. After incubation for 5 hr. there was little to choose between the three sets pre-heated for 1, 5, or 10 min.; all showed a higher proportion of germinations than the others. Then after 7 hr. there was a slight increase in numbers germinated in the set heated for 5 min., as well as a difference in the average dimensions of the young growing filaments. This difference in length was most noticeable in the series that had been heated for longer periods. Thus filaments 15–20 μ. long were conspicuous in the 10, 15 and 20 min. series, whereas lengths of 1–10 μ. were more general in the 100° for 1 min. set, as well as in the unheated control. An intermediate length of 5–15 μ. was found in the 100° for 5 min. set, where there were the greatest number of germinations. The values quoted were based on the average of ten readings for each slide. After incubation for 9 hr. small mycelia could be found in most slides; thereafter, the growth was too crowded for further observations to be made.

Examination of the cellophan growths by means of the 4 mm. dry objective with the phase-contrast system revealed no differences between the heated and unheated spores with respect to their germination, apart from the acceleration or retardation already mentioned. It was not possible to distinguish clearly the darkening of the spores because of the high degree of refractility of the cellophan itself. For this purpose the companion set of liquid cultures (sown with identical suspensions and incubated for the same periods of time) was used and several wet preparations were made from each tube with and without the addition of toluidine blue (see Methods). Similar results were found with unstained or stained material: all germinated spores, heated or unheated, exhibited a loss of refractility accompanied by an increase in permeability to dye. Owing to the tendency of the spores to show Brownian motion it was often found advisable to dry the fixed cells on the coverslip before mounting. In this way the differences in density between germinated and non-germinating spores lying in the same plane could readily be distinguished (Pl. 3, fig. 13). It was often possible to find numbers of darkened spores which showed no signs of germ-tubes, either because the specimen had been removed at an early stage in the course of the incubation (Pl. 3, fig. 12), or because the heat treatment (especially for 1–10 min.) had resulted in a preliminary 'quickening' of the spores followed by degeneration and death of large numbers of them (Pl. 3, fig. 16). The contrast between a mass of darkly staining heated spores (Pl. 3, fig. 16) and the clear refractile unheated spores (Pl. 3, fig. 15) was not always as clear-cut as in these photographs. Thus, clear unchanged spores were often found in heated material whether the period of heat treatment had been of short (Pl. 3, fig. 11) or of moderate (Pl. 3, fig. 14) duration.

Germination of spores subjected to heating for 30 *min. to* 4 *hr.*

In general, these longer periods of heating resulted in a higher proportion of the spores remaining unchanged. This is well seen in Pl. 4, fig. 17, where the approximately even distribution of clear and dense spores is visible over a broad field. But here again the germinated spores (around which small rectangles have been drawn) will be found stained with toluidine blue. This illustration is an example of the higher limits of thermotolerance (30 min. at 100°), beyond which normal growth in microculture rarely occurred within the first 12 hr. of incubation. Thus Pl. 4, fig. 18, depicts a 24 hr. growth of material which had been heated for 60 min. at 100°. The tendency towards rapid filamentous elongation on the part of those spores which did survive prolonged heating was very noticeable in the sets exposed to 100° for 30–40 min.; a typical example can be seen in that portion of a main filament which extends diagonally across the entire field in Pl. 4, fig. 17.

When very dense suspensions of all the stock strains were subjected to drastic heating (see Methods), it was found that five strains (D3, B2, H, D, M) were capable of producing typical surface growth within 24 hr. at 60° after being heated at 100° for periods of 40–100 min. In the case of strain H survivors were obtained after heating for 4 hr. at 100°. Random samplings during the early stages of growth revealed no perceptible differences as regards the aspect of the germinating spores, but when the drastically heated suspensions were plated it was often found that there were sectors devoid of aerial mycelium in the few giant colonies produced (Pl. 4, fig. 22). It was rarely possible to subculture from these sectors, such growths as ensued being of a transitory vegetative nature.

Internal structure of spores

Occasionally during the examination of living slide cultures, and more often in preparations of fixed and very lightly stained cells, amidst the general mass of homogeneous spores certain individuals were noticeable in that they seemed to have an eccentric 'nucleus' (Pl. 3, figs. 12, 16; Pl. 4, fig. 18). Some confirmation of this aspect was obtained by electron microscopy of normal unheated growths. Thus the immature spore attached to the parent stalk was found to be dense and impermeable (Pl. 4, fig. 19), while the mature spores varied from density (see two spores in top left-hand corner and centre of Pl. 4, fig. 20) to partial density (see bottom and right-hand spores in Pl. 4, fig. 20; also enlargement in Pl. 4, fig. 21).

Artificial darkening of spores

When suspensions of *Micromonospora vulgaris* spores were mixed with adenosine according to the methods used by Pulvertaft & Haynes (1951) with eubacterial spores, no artificial darkening of the spores occurred. Similarly, incubation of the spores with L-alanine as described by Powell (1950) for *Bacillus subtilis* did not cause changes in staining properties or loss of heat resistance in the case of *Micromonospora vulgaris*. It would therefore appear that the heat-resistant spores of *M. vulgaris*, although resembling eubacterial spores in refractility and relative impermeability, possessed these properties in differing degrees and possibly as the result of different mechanisms. In view of the entirely different mode of growth and of sporogenesis shown by the actinomycete, this is not unexpected.

This work was done by the author as a member of the scientific staff of the Agricultural Research Council. I wish to thank Professor J. Cruickshank, C.B.E., for the hospitality of his department; Dr D. M. Webley of the Macaulay Institute for Soil Research for permission to use the Cook, Troughton and Sims phase-contrast microscope and help with the photographs taken with it; and Miss Mildred Mackay for technical assistance. Mr Gard of the University of Aberdeen kindly took the electron micrographs.

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EXPLANATION OF PLATES

Phase-contrast preparations unless stated otherwise.

Plate 1

Fig. 1. Spores of *M. vulgaris* (strain B2) in slide culture before incubation. (×1325.)

Fig. 2. Enlargement of rectangle in fig. 1. (×4635.)

Fig. 3. Same as fig. 1, after 1 hr. at 60°. Observe darkening of certain spores. (×1325.)

Fig. 4. Enlargement of rectangle in fig. 3. (×4635.)

Fig. 5. Same as fig. 1, after 3 hr. at 60°. Note germ-tube issued by central spore. (×1325.)

Plate 2

Fig. 6. Enlargement of rectangle in fig. 5. (×4635.)

Fig. 7. Same as fig. 1, after 4 hr. at 60°. Germ-tube has produced filamentous branch. (×1325.)

Fig. 8. Enlargement of rectangle in fig. 7. (×4635.)

Fig. 9. Another field in same specimen after 6 hr. at 60°. Beginning of ramification. (×1325.)

Fig. 10. Same as fig. 9, after 24 hr. at 60°. Small mycelium. (×1325.)

Plate 3

Fig. 11. Spore suspension of *M. vulgaris* (strain D3) heated 5 min. at 100°, cultured 18 hr. in liquid medium. Note number of clear unchanged spores as well as young growths. (×1920.)

Fig. 12. Spores (strain D3) heated 15 min. at 100°, cultured 5 hr. in liquid medium. Dense spores, only one germinated. (×3120.)

Fig. 13. Spores (strain D3) heated 30 min. at 100°, cultured 18 hr. in liquid medium. Of two spores fixed in same plane on coverslip germinating one is dark, non-germinated, one clear. (×3120.)

Fig. 14. Spores (strain D3) heated 20 min. at 100°, cultured 6 hr. in liquid medium. Some clear and some dark spores still attached to parent branch; dense spore at top germinated. (×1920.)

Fig. 15. Impression preparation of mature 4-day growth (strain B2) on agar plate. Spores predominantly clear. (×2160.)

Fig. 16. Spores (strain D3) heated 10 min. at 100°, cultured 18 hr. in liquid medium. Stained toluidine blue. Young mycelial growth and mass of dense spores. (×2400.)

PLATE 4

Fig. 17. Spores (strain D3) heated 30 min. at 100°, cultured 18 hr. in liquid medium. Stained toluidine blue. Both clear and dark spores. Germinating spores (with small rectangles drawn round) always dark. Growing filaments showing marked elongation, the one crossing the field diagonally being only a short portion of the real growth. (×1920.)

Fig. 18. Spores (strain D3) heated for 60 min. at 100°, cultured 24 hr. in liquid medium. Stained toluidine blue. Light and dark spores, some of which give indications of internal structure. (×2400.)

Fig. 19. Immature spore (strain H) attached to parent stalk. Spore completely dense. Electron micrograph. (×17,000.)

Fig. 20. Mature spores (strain H) from same preparation as in fig. 19. Two spores completely dense, two showing apparent eccentric 'nucleus'. Electron micrograph (×6000.)

Fig. 21. Enlargement of partially dense spore in fig. 20. (×29,500.)

Fig. 22. Giant colony (strain H) with sector devoid of aerial mycelium and small secondary outgrowth. Survivor from spore suspension heated 80 min. at 100°. (×1.)

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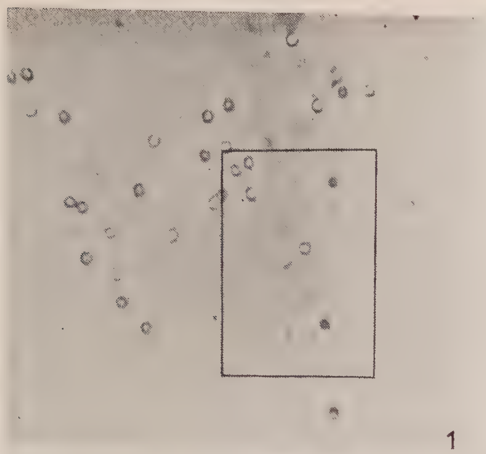
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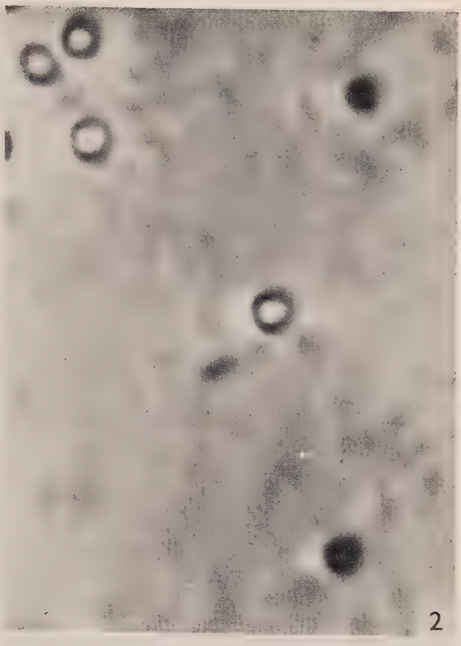
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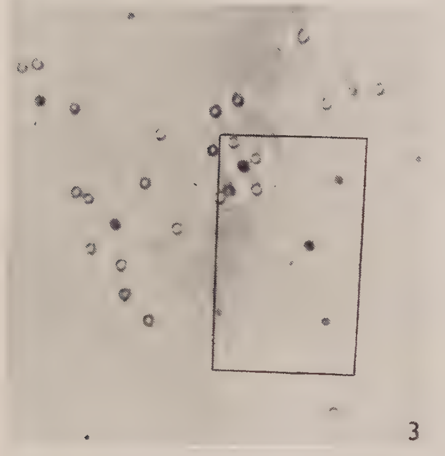
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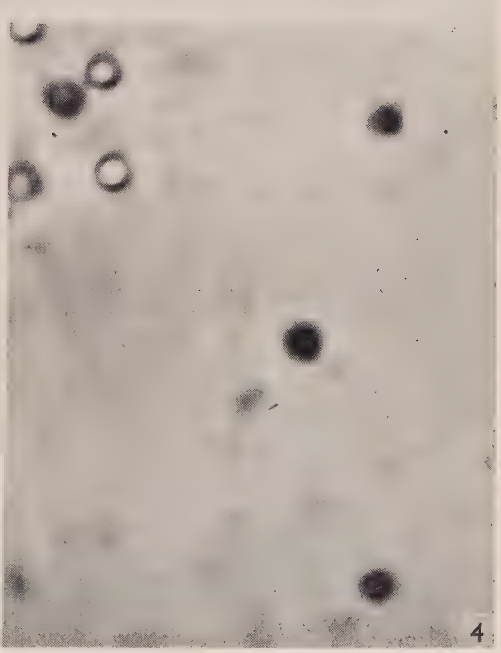
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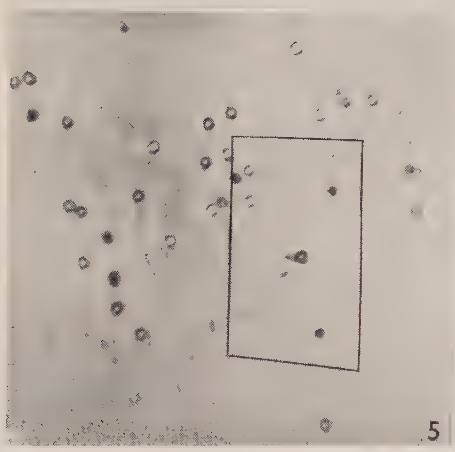
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D. ERIKSON—GERMINATION OF SPORES OF *M. VULGARIS*. PLATE 1

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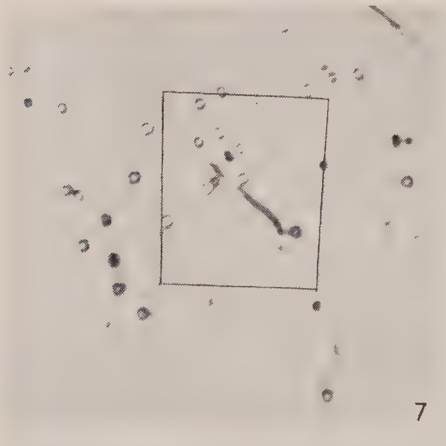
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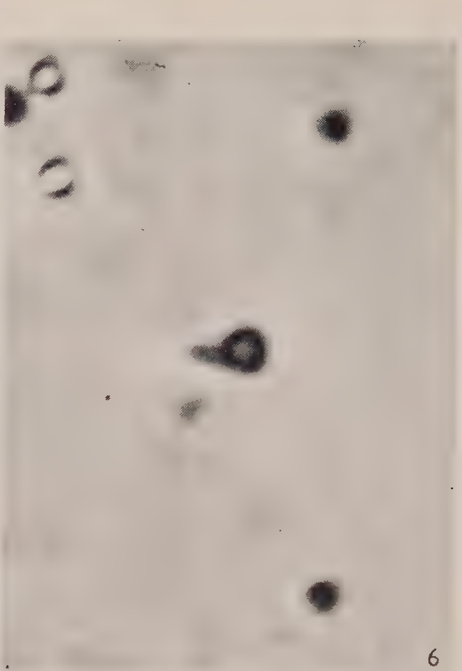
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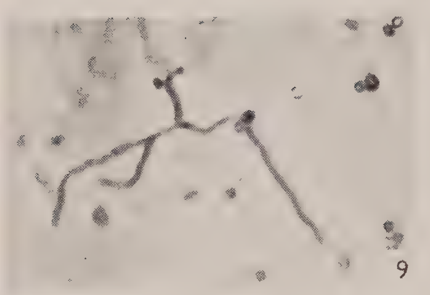
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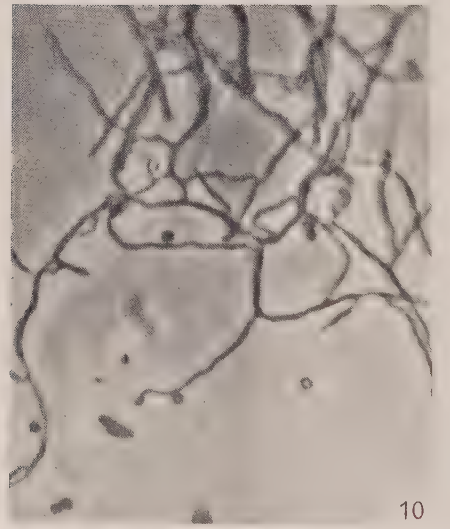
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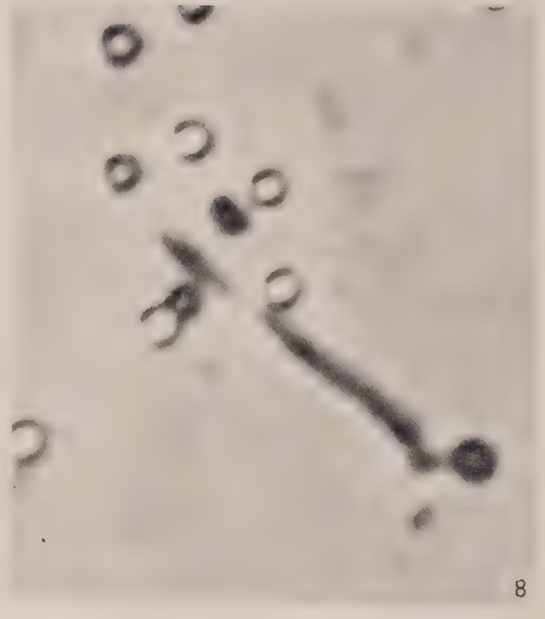
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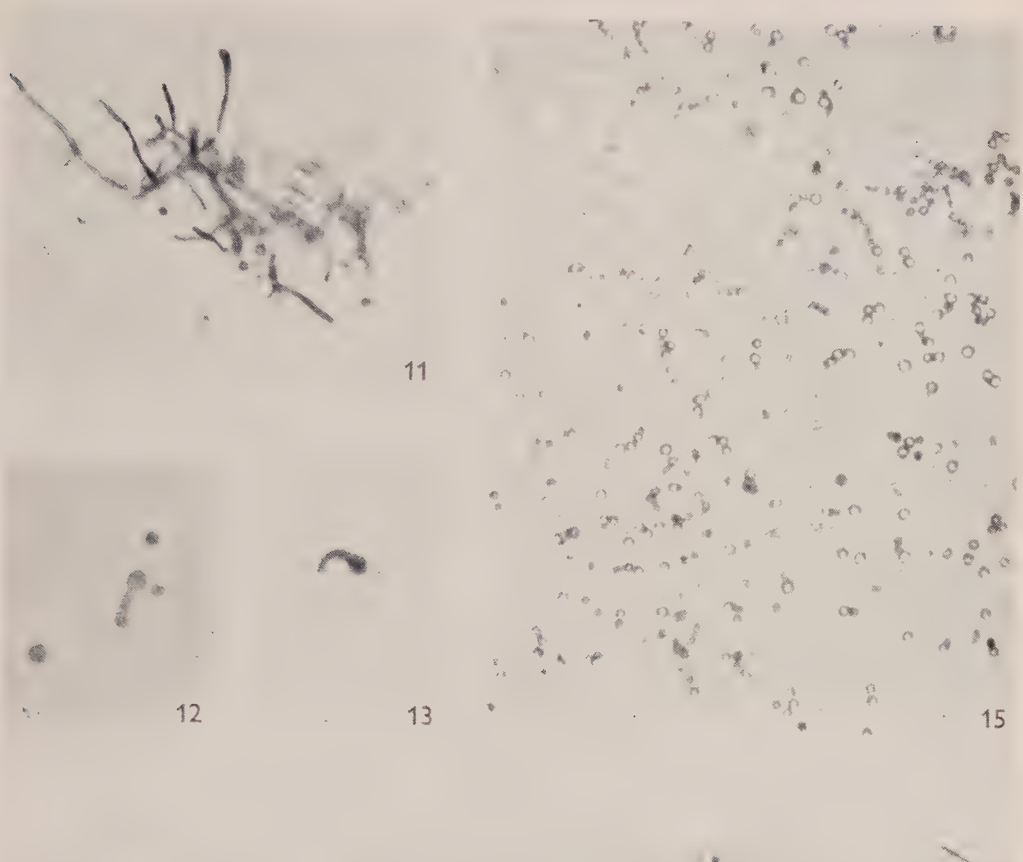
D. ERIKSON—GERMINATION OF SPORES OF *M. VULGARIS*. PLATE 2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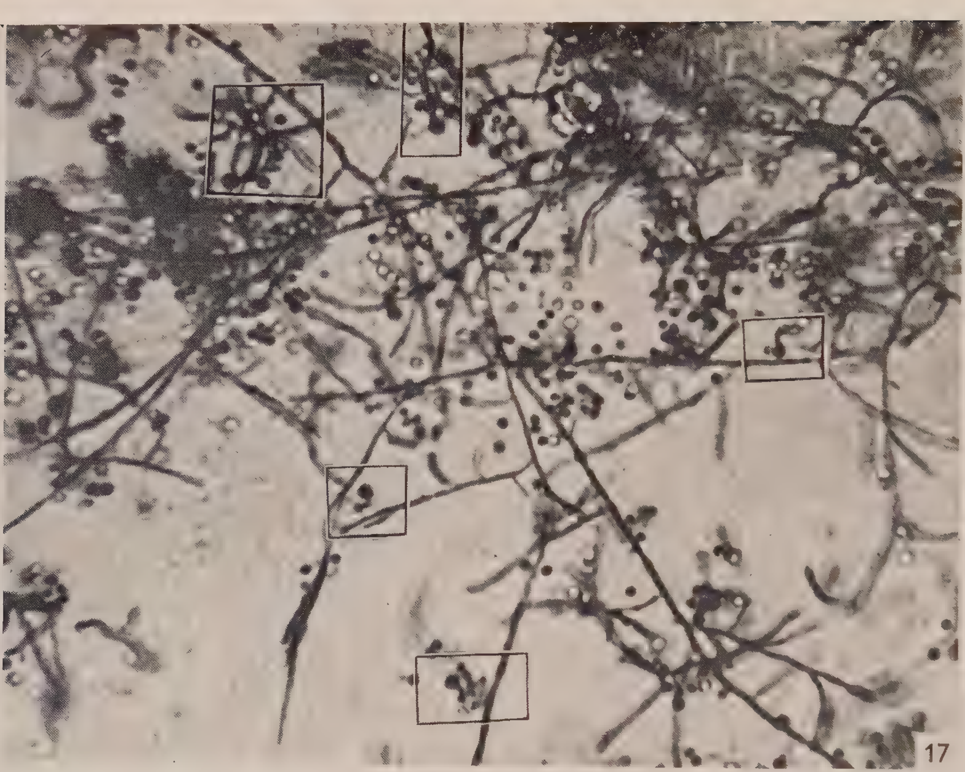
D. Erikson—Germination of spores of *M. vulgaris*. Plate 3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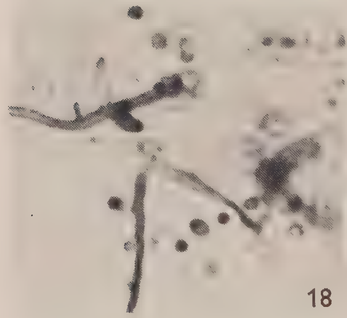
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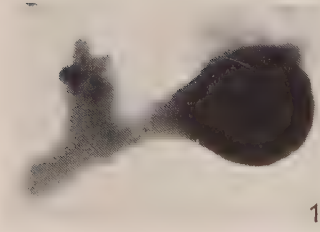
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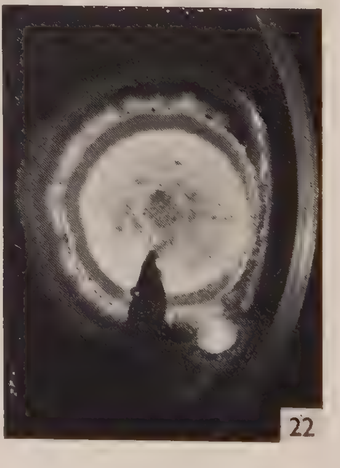
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D. Erikson—Germination of spores of *M. vulgaris*. Plate 4

Erikson, D. (1955). *J. gen. Microbiol.* **13**, 127–135

Thermoduric Properties of *Nocardia sebivorans* and other Pathogenic Aerobic Actinomycetes

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SUMMARY: The pathogenic partially acid-fast lipophilic organism *Nocardia sebivorans* and a closely allied species were found capable of withstanding exposure to 90° for 10 min. when dispersed in phosphate buffer suspensions in small sealed ampoules. Denser suspensions withstood 3 min. at 100°. The nature of the medium on which the cells were grown did not seem to influence their thermoduric properties. Of the two morphotypes of *N. sebivorans* that were originally isolated, one (G) consistently showed a higher degree of heat-resistance. The morphotypes were further distinguished by slight differences in the 'feel' of the cells and in their response to micromanipulative procedures. Different single cell isolates of the two morphotypes varied as regards thermotolerance, but the more resistant were G isolates. The lag period following heat treatment was generally accompanied by a compressed and stunted mode of growth, featuring prolonged angular division. Subsequent growth was normal.

Two strains of the pathogenic *Streptomyces madurae*, as well as two saprophytic soil species of *Nocardia*, were killed by exposures of 1–2 min. at 65°. An organism received as a blood culture and belonging to the *Streptomyces albus* group, was able to withstand 90° for 1 min. Subjection to heat treatment increased the inherent autolytic tendency of the culture and affected the type of sporophores produced during the first generation.

Apart from the true thermophilic organisms, which so far have been reported only within the genera *Micromonospora* (or *Thermoactinomyces* Tsiklinsky) and *Streptomyces*, the actinomycetes in general have been found to possess little resistance to heat. Waksman, Umbreit & Cordon (1939) tested over one hundred mesophilic sporing streptomycetes and found that the majority were killed by short exposures to temperatures as moderate as 50°, and that there was in fact a physiological line of demarcation between those strains capable of living at elevated temperatures and the common run of ordinary streptomycetes isolated from composts, soil and other natural products. Other investigators have agreed that typical streptomycete spores differ from eubacterial spores in their lack of heat-resistance.

Few attempts have been made to test the thermotolerance of non-sporing representatives of the genus *Nocardia*. Sforza (1940, 1946) in Ethiopia described under the generic name *Asteroides* Leonardi & Puntoni several organisms isolated from cattle farcy and mastitis, which appear very similar to members of the species-groups *Nocardia farcinica* and *N. asteroides*. Certain of these pathogens, notably *Asteroides granulosus* Sforza, were capable of withstanding 60–65° for 30 min. when dispersed in sterile milk. They were therefore able to survive certain pasteurization processes. Gorrill & Heptinstall (1954) noted the ability of *Nocardia sebivorans*, an organism pathogenic to experimental animals, to withstand 60° for 30 min. Both *N. sebivorans* and the organism described by Sforza are partially or entirely acid-fast, and belong to the more stable or 'hard' mycelial section of the genus *Nocardia*. *N. sebivorans* is a lipophilic organism, utilizing paraffin and other fatty substances, and produces a relatively abundant aerial mycelium. In these respects it compares with *N. paraffinae*, a common soil saprophyte. These firm-textured, well-ramified species producing considerable aerial growth (Ørskov, 1923, group II*a*) have the closest morphological affinities with the genus *Streptomyces*. On the other hand, those streptomycetes which have lost their power to produce aerial mycelium can readily be confused with species of *Nocardia* until a more careful examination is made (cf. Waksman, 1953, p. 1).

In the present work the thermoduric properties of single cell isolates of two morphotypes of *Nocardia sebivorans* were tested and compared with those of a closely related form (strain El1, isolated by Dr Elek of St George's Hospital Medical School, London, from a fatal case of meningitis). *N. paraffinae* (NCTC no. 3422) and an allied form (strain BSN, isolated by Dr D. M. Webley, Macaulay Institute for Soil Research, from beach sand) were included in the preliminary tests, but failed to show sufficient degree of thermotolerance to justify continued study. In addition, three cultures of streptomycetes from morbid material were tested: two strains of *Streptomyces madurae* (Th/1 and Th/2, isolated by Major Altmann of Tel Hashomer Hospital, Israel, from mycetoma of the foot); and a form of *S. albus* (no. 1569, also isolated by Major Altmann from blood), which resembled an organism (NCTC 4572) described by Erikson (1935) as *Actinomyces bovis* Harz var. *nigerianus*. All three cultures were non-acid-fast and proteolytic, thus differing essentially from the nocardial species. The two strains of *Streptomyces madurae* produced very little aerial mycelium and that only on simple substrates, and failed to show any high degree of thermotolerance. *S. albus* 1569 readily sporulated and was found more suitable for testing in conjunction with *Nocardia sebivorans*. This organism also possessed autolytic properties.

METHODS

Fifteen single-cell isolates of *Nocardia sebivorans* were prepared according to the methods of Erikson & Masson (1954): six from the morphotype G (sem, vi, mb, mg, lmg, sg); and nine from the morphotype W (2a1, a, 3, b, 22, c, 31, d, 1). Descriptions of the original morphotypes are given by Erikson in an addendum to Gorrill & Heptinstall (1954).

Cells for heat testing were harvested from the various isolates of *Nocardia sebivorans* and of strain El1 as follows: washings were taken from 3-, 5-, 7- and 10-day growths on: (*a*) nutrient agar slopes; (*b*) surface of nutrient broth in 1 oz. stoppered bottles; (*c*) cellophan over nutrient agar in poured plates. They were then shaken in phosphate buffer, lightly spun, and relatively smooth suspensions prepared in the manner described by Webley (1954*a*) for *Micromonospora vulgaris*. Preliminary thermal death-rate experiments, using dilute suspensions in sealed ampoules in the manner previously used for the thermophilic micromonosporas (Erikson, 1952), yielded such irregular results as to make it clear that the samples of the suspensions employed were not comparable as regards their content of thermotolerant cell elements. This was the case whatever the age of the culture on the complex nutrient stock medium, and variations in the technique of preparing the suspensions made little difference. Accordingly, much denser suspensions were employed than in the case of the spore-bearing thermophile.

Isolates of *Nocardia sebivorans* and of strain E11 were also grown in an ammonium phosphate + Czapek salt basal medium with the addition of solid or liquid paraffin, or with the Na salts of valeric, caprylic or butyric acids. Growth was good but slow in most cases, and such media could not compare with the stock nutrient agar or broth for the purpose of collecting cells. Suspensions were made from a set of 2-month old cultures with solid paraffin as the source of carbon.

The following procedures were adopted: in the case of growths under conditions (*a*) and (*c*), either two well-grown slopes or one cellophan circle yielded sufficient material for 10 ml. of phosphate suspension to be subdivided in 0·5 ml. lots in sealed ampoules; in the case of growth under condition (*b*), the surface growth from a 1 oz. bottle was resuspended in 2 ml. phosphate buffer, and a separate bottle employed for each heat test. The sealed ampoules were totally immersed in a water-bath, and heated for 1–10 min. at temperatures rising in 5° steps from 60° to 95°. The 1 oz. bottles containing primary suspensions were heated at 100° in a steamer for the same times. Allowance was made for the time taken for the contents of the vessels to reach the desired temperature, and at the expiry of the period of heating all vessels were placed in ice. The contents were then removed to sterile Durham tubes, centrifuged to remove the suspending fluid, and 0·5 ml. (or 2 ml. in the case of the screw-capped bottles) of a starch (1 %) + casein hydrolysate (0·1 %) + Czapek salts medium added. It was found that this medium was superior to nutrient broth in yielding a consistently higher proportion of survivors. The duplicate unheated controls were treated in the same way.

The cultures *Streptomyces madurae* (Th/1 and Th/2) and *S. albus* 1569 grew more slowly than the nocardial species and in the typical compact streptomycete fashion which made their dispersion more difficult. They were employed only in dense primary suspensions in small bottles, as described above. The sporulating *S. albus* 1569 was subject to autolysis on rich media. This was less evident when starch was incorporated in the medium. The CPS medium employed for the thermophilic micromonosporas (Erikson, 1952) was found suitable for the cultivation of all three organisms.

Slide cultures were prepared from the heated and unheated suspensions in the same way as for the germination of heat-resistant spores of *Micromonospora vulgaris* (Erikson, 1955). The mode of growth was observed directly at frequent intervals by means of phase-contrast microscopy. Representative sets of heated cultures in Durham tubes were also removed at intervals during the course of incubation, and the cells stored in 5 % neutral formalin. Incubation throughout was at 37° except for strain BSN and *Nocardia paraffinae* (20–23°).

RESULTS

Thermotolerance of different species

Nocardia sebivorans and strain E11 withstood a maximum exposure of 10 min. at 90° when dispersed in 0·5 ml. lots in small sealed ampoules, and of 3 min. at 100° in denser suspensions in screw-capped bottles. Survivors were obtained from mature growths on nutrient broth, nutrient agar, and the various ammonium salt media with fatty acids or paraffin added. The maximum tolerated exposure for *Streptomyces albus* 1569 was 90° for 1 min. The two strains of *S. madurae* were much less resistant, their maximum being 1–2 min. at 60°. A similar degree of thermotolerance was shown by the soil saprophytes BSN and *Nocardia paraffinae*, which survived 1–2 min. at 60° but were killed by 1–2 min. at 65°.

Thermotolerance of different isolates of Nocardia sebivorans

It was stated earlier (Erikson, in addendum to Gorrill & Heptinstall, 1954) that two distinct morphotypes appeared spontaneously within this culture when first studied. On the original rich complex organic media on which they appeared the two types were macroscopically distinct; W constantly produced a prolific aerial mycelium which made the colonies seem chalky white (Pl. 1, fig. 1), whereas the aerial growth of G was much less developed giving a grey aspect to the culture. These distinctions persisted after animal passage, although no qualitative difference could be found between the lesions induced by the two growth-forms.

Such gross visible differences in growth-form were not very marked after the two types had been maintained for some time on simpler media, although a readier development of aerial mycelium could usually be detected on the part of W when growing in microculture (see Pl. 1, fig. 6). Moreover, during the processes of micromanipulation (Erikson & Masson, 1954) it was repeatedly found that there were perceptible differences in the behaviour, refractibility, and 'feel' of the cells of G and W. Thus G cells from nutrient broth cultures were difficult to see in micro-droplets, proved almost intractable by appearing to have a surface coating that resisted seizure by micro-instruments, and were pronouncedly lipophilic—large numbers passing rapidly into the oil phase. This was also the case, although to a lesser extent, even when they had been cultivated for several subcultures in an aqueous Czapek glucose nitrate medium, whereas such treatment tended to render the W cells very much more manageable.

Again, while both G and W isolates were capable of growing in droplets of Czapek mineral medium oil without added carbon source and of extending their filaments into the surrounding oil of a de Fonbrune paraffin oil micromanipulating chamber, the more vigorously growing W isolates were more consistent in producing a clearly marked zone of gas bubbles in the oil (Pl. 1, fig. 9). That these bubbles were not air bubbles introduced with the droplet of medium was proved by repeated careful experiment. This phenomenon was of unusual interest, since visible gas production is very rare among actinomycetes. The G morphotype, partly perhaps because it did not grow so well in these circumstances and passed into the oil phase earlier and more completely, seldom produced this display of bubbles. When an immersion oil (polyric oil—see Erikson & Masson, 1954) was substituted for paraffin, the bubbles were not apparent even in the case of W and the filamentous growth was noticeably more elongated.

These slight distinctions between the G and W types were borne out by variations in their responses to heat treatment. Since the early production of an aerial mycelium by the surface growth on liquid media is a group characteristic of this type of nocardia (see Erikson, 1935), the time taken for its appearance after the heating of the cells was regarded as an index of recovery on the part of each isolate. Thus when three G and three W isolates, together with some strain E11 organisms, were heated for 3 min. at 70°, 85° and 100°, respectively, surface growth with aerial mycelium appeared after 2 days of incubation in strain G/lmg and to a lesser extent in strain G/sg in the 85° series; after 3 days with the same two isolates in the 100° series; and it was not until after 7–10 days of incubation that the bottom growth in strains E11 and W/22 gave rise to surface growth with aerial mycelium in the 100° series. In the control series, typical surface growth regularly appeared within one day.

As was pointed out in Methods, quantitative measurements were not readily applicable to the suspensions used for these heat tests. In practice it was found that the most consistent results were obtained when the heated suspension was distributed in a number of small Durham tubes and relative comparisons made over a large number of tubes. Few differences could be detected in the survival capacities of the different isolates for exposures up to 3–5 min. at 70°, but thereafter strain G/lmg constantly showed surface growth before the other isolates. In all the tests three G isolates (lmg, sg, vi) survived exposure to the more elevated temperatures more frequently than any of the W isolates.

Mode of growth of heated and unheated cells of Nocardia sebivorans *and other nocardias*

As will be seen from Pl. 1, fig. 1, mature growth of the W strain of *Nocardia sebivorans* is as a rule so well covered with aerial mycelium that suspensions of cells washed from the surface will consist mainly of the components of that aerial covering. The characteristic angular fashion in which these aerial strands develop in maturity is shown in the impression preparations of strains G/sg and E11 in Pl. 1, figs. 2 and 3, respectively. Segmentation within the long filaments is visible in Pl. 1, fig. 2, and many of the fragmented cylindrical cells can be seen in Pl. 1, fig. 3. The germination of these cells as of other mycelial fragments follows the nocardial pattern (Ørskov, 1923; Erikson, 1935; McClung, 1949). Swelling of the primary cell is followed by extrusion of a short tube which often immediately changes its orientation before segmenting or branching, while at the obtuse angle so formed the primary cell may develop one or more large round cells. Each of these round cells may behave in a similar fashion with the result that there is an outstanding impression of polymorphy and of short cells in angular apposition during the early stages of growth (see Pl. 1, fig. 4). In the subsequent stages under normal conditions this appearance gives place to the typical branching mycelium of an actinomycete (Pl. 1, fig. 5), but with the early development of aerial buds from the segmented filaments an angular aspect may again be introduced (Pl. 1, fig. 6). Prolongation of this angular mode of development was found to be a feature of the lag period which followed heat treatment of the cells. The samples of 2-day bottom growth shown in Pl. 1, fig. 7, illustrate the tardy development of cells which repeatedly changed their orientation with each subdivision and which seemed to suffer from inhibition of the factor which permitted undeviating growth along one axis resulting in filaments. The cells giving rise to this growth were subjected to the upper limit of heat treatment: 100° for 3 min.

These phenomena were found in general in the early stages of development of cells which had been held at temperatures of 75° and over. The deleterious effect of this heat treatment did not appear to be permanent, and on prolonged incubation luxuriant surface growth with characteristic aerial mycelium was obtained with all survivors, although this might sometimes require as long as 7–10 days. With the less drastically heated cells (60° for 15 min.) there was often little difference to be found in the mode of growth of the heated cells (Pl. 1, fig. 11) compared with that of the control (Pl. 2, fig. 12). After exposure to intermediate temperatures (e.g. 80° for 1 min.), there was often a more prolonged persistence of the short round cells (Pl. 2, fig. 13, bottom right-hand), which commonly occur only during the initial stages of growth (cf. Pl. 1, fig. 4).

Abnormally large round, hexagonal, clavate, or quite irregular cells with thickened walls (Pl. 1, fig. 8) appeared in paraffin wax cultures and also on *n*-dodecane (cf. with growth of *Nocardia opaca* on waxes, etc.: Webley, 1954*b*). No enhancement of heat resistance was shown as the result of culturing *N. sebivorans* and strain E11 on these fatty substances; and therefore no attempt was made to defat the cells before heating. The grotesque cells were proved viable and capable of producing normal growth when seeded on to the surface of slide cultures with ordinary media.

Preliminary tests with the two saprophytic soil species revealed only a limited degree of thermotolerance—60° for 1–2 min. It should, however, be borne in mind that their optimum temperature for growth was 19–23°, as against 37° for the pathogenic species. Neither *Nocardia paraffinae* nor strain BSN lost the capacity of producing aerial growth when subjected to this moderate degree of heat (Pl. 1, fig. 10), and the chief response was that of retardation of growth.

Mode of growth of heated and unheated Streptomyces albus 1569

In its early mycelial stages *Streptomyces albus* constantly showed the characteristic uniformity of a streptomycete as regards filament width and monopodial branching. In Pl. 2, fig. 14, it will be noted that in several places the filaments approaching from neighbouring growths turn away from one another and that, as Erikson (1949) stressed, there are no clear cases of anastomosis in the vegetative mycelium. The characteristics of the subsequent aerial growth were tightly coiled sporophores (Pl. 2, fig. 16) on a good agar medium such as CPS, but with long straight interconnecting strands between the growths (Pl. 2, fig. 15). The tight spirals became rarer and straight spore-chains more common in the surface growth on liquid media of simpler composition, e.g. 5% casein digest with different sugars (Pl. 2, figs. 17–19).

Subjection to heat treatment emphasized the inherent tendency of the organism towards autolytic disintegration. An extreme degree of segmentation (Pl. 2, fig. 20) followed by partial autolysis was frequently seen in the 1- to 3-day vegetative growths on slide cultures which had been sown with cells heated for 1 min. at 80°. Similar cells were able to produce normal surface growth in liquid media, but when they were heated for 1 min. at 100° only bottom growth of the puffball colony type appeared in the course of 3 days. Then on the fifth or sixth day the survivors (usually only 1–2 colonies/tube) were no longer visible because of autolysis. Yet some of the mycelial fragments remained viable although the liquid generally appeared quite clear, for when this was added to fresh medium normal growth very slowly (15–20 days) developed again.

The aerial growth produced after exposures to 80° for 1 min. (the upper limit for the production of surface growth after heat treatment) was generally short and straight, and the sporophores were often sterile. When the exposure was more moderate, e.g. 60° for 1–5 min., normal spiral sporophores were frequently produced. None of the abnormalities noted after heat treatment persisted in subculture.

DISCUSSION

From a phylogenetical point of view the most interesting findings in this work were that the nature of the injury due to heat varied with the type of organism heated, and that in each case there was temporary loss of the ability to produce a complex morphological structure characteristic of the particular organism. In the case of the heated *Nocardia sebivorans* organisms, inhibition of aerial mycelium production combined with a prolonged angular development of the vegetative mycelium resulted in growths morphologically similar to those of less developed nocardial species in a different systemic subdivision. The differences in thermotolerance between the two morphotypes seemed correlated with slight variations in the rate of growth and properties of the external membranes of the entire organism and could not be attributed to any particular types of cells. It was noteworthy that none of the thick-walled polymorphous cells produced on media containing waxy substances showed any superior resistance to heat.

In the case of the sporing *Streptomyces albus*, the effect of heat on the production of aerial mycelium was complicated by enhancement of the autolytic tendency of the strain. Yet a similar patter prevailed. Instead of spiral sporophores—the most advanced type of structure—straight aerial filaments, similar to those produced normally on certain poorer media, were commonly obtained after heat treatment. Often these sparse aerial filaments were sterile, and when accompanied by an extreme degree of segmentation of the vegetative mycelium the entire cell complex resembled that of a nocardial type.

This work was done by the author as a member of the scientific staff of the Agricultural Research Council. I am indebted to Professor J. Cruickshank, C.B.E., for the hospitality of his department; Miss F. M. Masson of the Rowett Research Institute for Animal Nutrition for help with the micromanipulations; and Miss Mildred Mackay for technical assistance.

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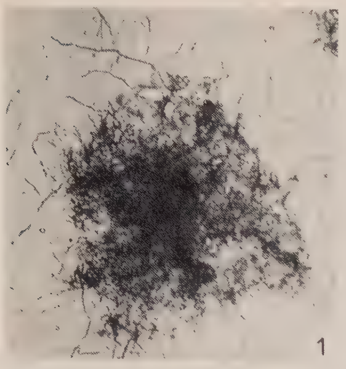

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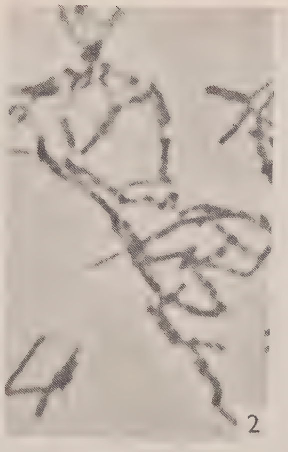

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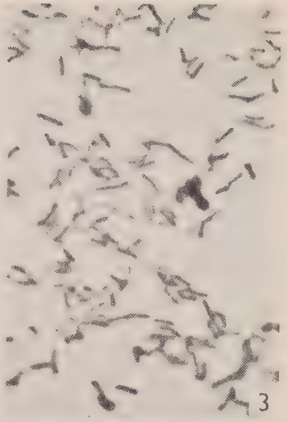

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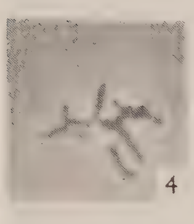

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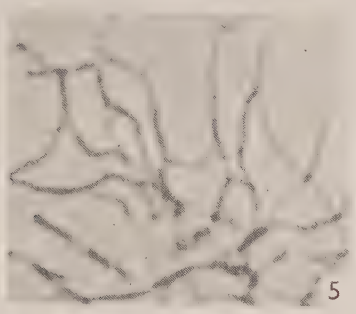

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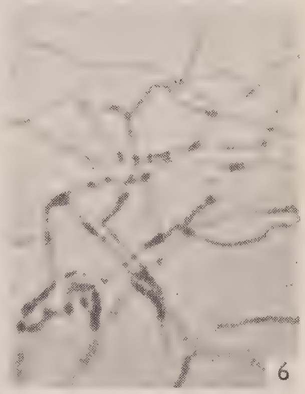

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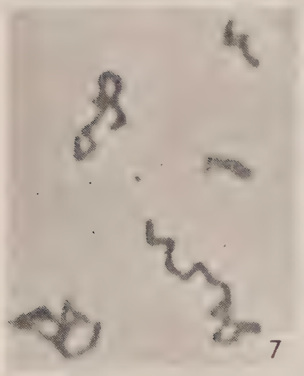

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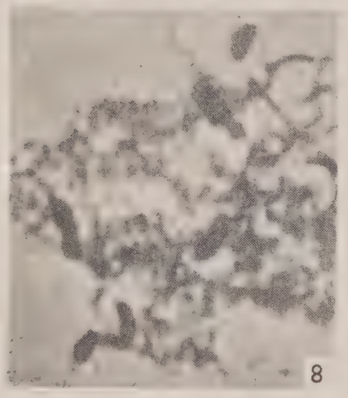

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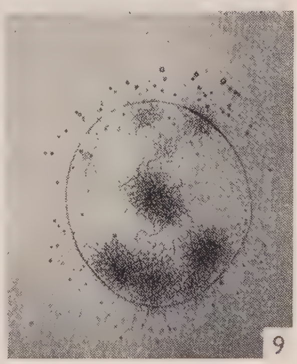

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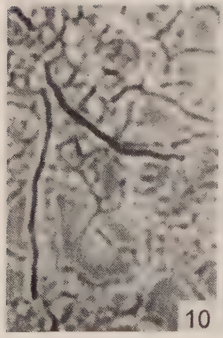

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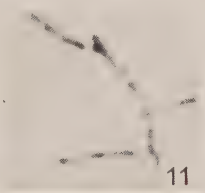

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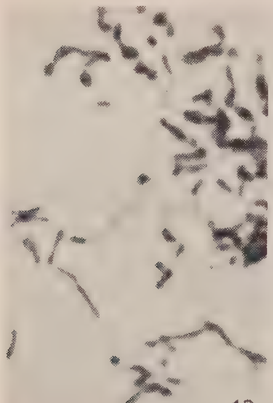
D. ERIKSON—THERMODURIC AEROBIC ACTINOMYCETES. PLATE 1



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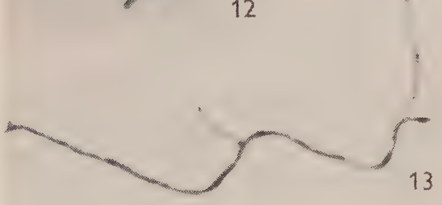

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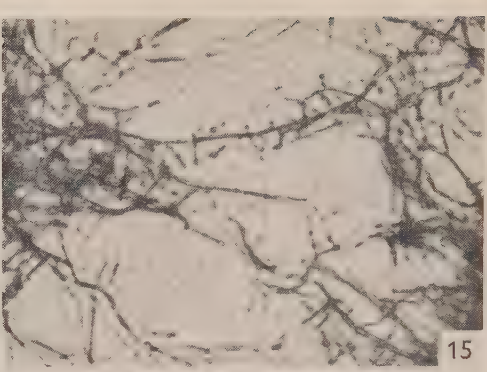

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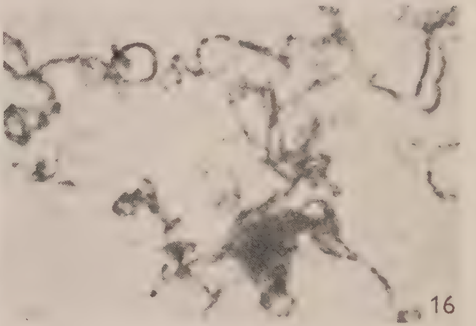

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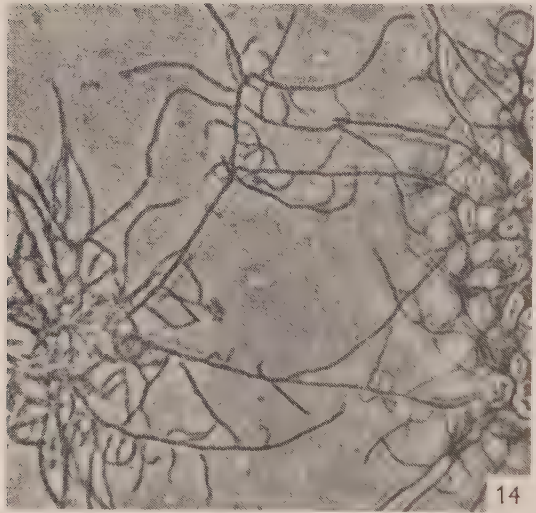

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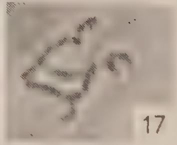

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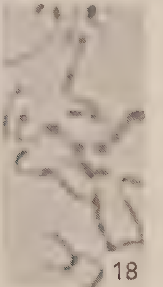

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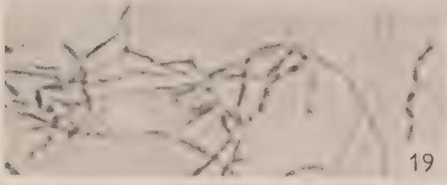

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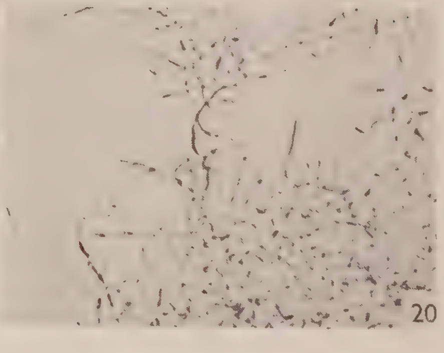

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D. Erikson—Thermoduric aerobic actinomycetes. Plate 2

EXPLANATION OF PLATES

Phase-contrast preparations unless otherwise stated.

Plate 1

Fig. 1. Mature colonies of *Nocardia sebivorans* (type W) with good development of aerial mycelium. (×150.)

Fig. 2. Impression preparation of mature aerial mycelium, strain G/sg, 2 weeks on nutrient agar, showing angular development of aerial strands and advanced stage of segmentation. (×2160.)

Fig. 3. Impression preparation of aerial growth, 2 weeks on nutrient agar, strain El1, fragmented cells, many still showing angular division. (×2160.)

Fig. 4. Slide culture, 16 hr. on nutrient agar, strain G/mb, showing swollen primary cells and change of orientation in young filaments. (×2160.)

Fig. 5. Young typical branching mycelium, strain W/1, 24 hr. on nutrient agar. (×1195.)

Fig. 6. Another portion of same specimen as in fig. 5, showing segmentation of filaments and early development of serial buds, giving angular aspect to growth. (×1195.)

Fig. 7. Strain El1, incubated for 48 hr. in starch casein hydrolysate liquid, after heating at 100° for 3 min., showing slow growth and marked angular development. (×2160.)

Fig. 8. Strain El1, 2 months on ammonium phosphate paraffin wax showing large clavate cells and normal filaments attached to fragment of paraffin wax. (×2160.)

Fig. 9. Droplet of Czapek salts, without added carbon source, in paraffin oil chamber of micromanipulating slide, strain W/1, after 3 days' incubation, containing several colonies derived from pool of short cell elements, demonstrating zone of gas bubbles in oil phase. (×60.) Photograph by Miss F. M. Masson.

Fig. 10. Strain BSN, grown at room temperature for 3 days after heating for 1 min. at 60°, showing segmenting growth on cellophan over nutrient agar, with two aerial filaments arising from centre of growth. (×1195.)

Fig. 11. Strain W/1, 24 hr. incubation at 37° after 15 min. at 60°. Slender branching filaments.

Plate 2

Fig. 12. Strain El1, 24 hr. incubation at 37° after 1 min. at 80°. Multiplication of short irregular cells. (×1195.)

Fig. 13. Strain W/1, 24 hr. incubation at 37° of unheated control. Very long slender branching filaments arising from primary cell. (×1195.)

Fig. 14. *Streptomyces albus* 1569. 24 hr. on cellophan over CPS agar. Ramifying mycelia with tips of filaments frequently turning away from adjacent filaments. (×930.)

Fig. 15. Another portion of growth in fig. 14, after 3 days, showing straight interconnecting aerial strands between the colonies. (×930.)

Fig. 16. Slide culture of 1569, 1 week on CPS agar showing individual coiled sporophores. (×1195.)

Fig. 17. Sporulating surface growth of 1569 in liquid glucose casein digest, 2 weeks, showing portion of loose spiral. (×2160.)

Fig. 18. Similar sporulating growth from surface of starch casein digest liquid medium, 2 weeks. Tightly coiled spiral sporophores rarer. (×2160.)

Fig. 19. Sporulating surface growth from melibiose casein digest medium, 2 weeks. Many long and mostly straight spore chains. Lightly stained with dilute toluidine blue. (×2160.)

Fig. 20. Vegetative growth, 2 days on cellophan over CPS agar after 1 min. at 80°. Extreme degree of segmentation in entire mycelium. (×720.)

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Loss of Aerial Mycelium and other Changes in Streptomycete Development due to Physical Variations of Cultural Conditions

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SUMMARY: Several isolates of monosporous lines of *Streptomyces coelicolor*, both normally pigmented and colourless variants, were obtained by microdissection of young vegetative mycelia. In each case their properties largely resembled those of the parents. This material was studied together with three strains of *Streptomyces scabies* of varying degrees of virulence, which had been isolated from potato scab lesions. Modifications of growth were noted with respect to gross colonial morphology on agars containing detergents, structure of aerial mycelium arising from various types of surfaces, and development of germinating spores in liquids and in contact with non-nutritive plane surfaces. In both species-groups those organisms which produced least aerial mycelium on stock nutrient agar were most susceptible to the inhibitory influence of 0·01 % Gemex 29 on the development of aerial mycelium in simpler media. Distorted aerial growths on detergent agar resembled irregular swollen sporogenous filaments produced in pectin + ammonium salt liquid media. These were also reminiscent of cases of sporogenesis in submerged cultures described by other workers. Apart from surface tensions operating at the air/medium interface, the relative degree of humidity in the culture vessel was found to have some effect upon variation of spore size. Germinating spores and young filaments growing in contact with plane surfaces were more prone to elongation than those growing in liquids. Branching was also influenced to a considerable extent by the nature of the physical environment.

In a recent review Hendlin (1954) pointed out that inadequate attention has in the past been paid to the profound effect which the physical environment must have upon the nutrition of micro-organisms. Such a viewpoint, which was restricted by Hendlin mainly to a consideration of such physical factors as temperature, pH values, oxidation-reduction potential, and CO_2 tensions in so far as they cause perceptible nutritional differences, can almost certainly be extended to other aspects of microbiology. Thus the alteration of the energy relationships at interfaces which is brought about by the addition of surface-active agents to the media in which various acid-fast bacteria are grown is a recognized method of altering the character of the growth of the organisms (Alexander & Soltys, 1946). Increased cell division and smaller cells result from the incorporation of Tween 80 in the media in which the non-acid-fast *Nocardia turbata* (Erikson, 1954) is cultivated. A certain degree of correlation has been noted between decreased humidity on the one hand, and increased production of sporogenous aerial mycelium by streptomycetes on the other (von Plotho, 1940; Erikson, 1947), suggesting that important phases in the development of a complex organism may be favoured or completely suppressed by fluctuations in the physical environment.

In the course of regular platings of monosporous lines of an agar-digesting strain of *Streptomyces coelicolor* on a carbohydrate-free Czapek mineral salt agar medium, it was noted that the frequency of non-pigmented or asporogenous sectors was greatly increased by the addition of small quantities of non-toxic surface-active agents. It was further found that some of the changes in aerial mycelium brought about in this way resembled those occurring in liquid cultures containing pectin as a source of carbon. The present work is a comparative study of the differing details of growth shown by representatives of the species-groups *S. coelicolor* and *S. scabies* in a pectin + ammonium salt liquid medium and aqueous Czapek nitrate medium containing insoluble chitin; on plane and non-nutritive surfaces such as glass coverslips and cellophan films superimposed on different nutrients; and in agar media with and without the anionic detergent Gemex 29.

ORGANISMS AND METHODS

The representatives of the two common species-groups which were used were as follows.

Streptomyces coelicolor. Strain A3 (2): descendant of a single spore isolate (Erikson, 1948), possessing characteristic agar softening and pigment producing properties and bearing spirally coiled aerial sporophores. Strains A3 (2)/1–15: the progeny of 15 mycelial fragments dissected by means of a micromanipulator from a 24 hr. vegetative microcolony of A3 (2); all possessed apparently similar properties to the parent. Strain 12b: a colourless variant, possessing little or no power of softening agar, having more or less straight aerial sporophores. Strains 12b/1–36: the progeny of 36 mycelial fragments dissected by means of a micromanipulator from a 24 hr. vegetative microcolony of 12b; all showed properties similar to the parent.

Streptomyces scabies. Strain Sc10: highly virulent to potatoes, producing a dark brown soluble pigment in protein media; strain Sc5: moderately virulent, also pigment producing; strain Sc4: not producing pigment in protein media but occasionally in pectin, weakly virulent. Strain Sc4: the most prolific in its production of a colourless to grey aerial mycelium on most substrates, but all strains capable of aerial growth, the individual sporophores being wavy to coiled. All these strains were isolated from potato scab lesions by Mr W. McKee of the Potato Storage Research Station, Loughborough, Leics., and kindly sent by him to me. The *S. scabies* strains were received in the freeze-dried state, and not opened until used for this study. The *S. coelicolor* strains were stored for 4 years in sterile soil after being prepared by micromanipulative methods as described.

The following media were employed:

Liquid media. A basal medium of Czapek mineral salts containing sodium nitrate with insoluble chitin (British Drug Houses Ltd.) as carbon source. This would not cause any alteration of surface tension even on prolonged incubation; or with the addition of 1 % (w/v) of soluble starch or a sugar, e.g. sucrose, glucose. A pectin medium containing citrus pectin (Edward

Gurr) and sodium ammonium phosphate prepared according to the method of Oxford (1944).

Solid media. The basal Czapek mineral salt medium containing 1 % sucrose was solidified with 2 % agar and combined with different sources of nitrogen: sodium nitrate, ammonium phosphate, sodium ammonium phosphate (all 0·1 %), or urea (0·5 %). When plating with these media duplicates were poured, and to one set was added 0·01 % of the detergent Gemex 29 (General Metallurgical and Chemical Industrial Ltd., London), which had been sterilized separately. Stock sporing growths used for inoculating the various types of cultures were maintained on 7–10-day slopes of starch (1 %) + casein hydrolysate (0·1 %) agar medium.

Slide cultures. These were prepared according to the methods described for the germination of spores of *Micromonospora vulgaris* (Erikson, 1955*a*). Spores of the strains being studied were seeded directly on to the agar surface or on to superimposed cellophan films, both with and without the addition of coverslips. Various media described above were used together with the stock nutrient (peptone + Lab Lemco + Marmite) agar.

The pH values of all media were at 7·0–7·2. Incubation throughout was at room temperature (19–23°). Observations were made by bright field or phase-contrast microscopy at daily or frequent intervals according to the progress of the growths.

RESULTS

Gross colonial morphology in sucrose + mineral salt agar medium incorporating Gemex

With urea as source of nitrogen. A representative series of colony-types has been selected from the large variety produced, and can be classified as those completely devoid of aerial mycelium and those where a slight aerial growth occurs centrally, peripherally, or in sectors. Of the *Streptomyces scabies* strains Sc10 most frequently exhibited a smooth piled up aspect; strain Sc5 a coherent skin-like growth with small colonies at the periphery, all entirely devoid of aerial mycelium, or with some slight marginal aerial mycelium; while strain Sc4 was the most consistent in producing a few sectors of aerial growth. *Streptomyces coelicolor* A3 (2) and all other isolates producing blue pigment grew abundantly to form a heavy corrugated membrane which lacked pigment and aerial mycelium. The colourless strains 12b/1–36 were least affected and grew moderately with a fair covering of aerial mycelium. In the control plates without Gemex growth was good throughout the series, and all colonies were evenly covered with their specifically typical aerial mycelium. When sucrose was omitted, there was poor growth in all cases except A3 (2) which showed the customary depressions round the colonies together with a normal development of aerial mycelium.

With sodium nitrate, ammonium phosphate, or sodium ammonium phosphate as source of nitrogen. The relative susceptibilities of the different strains to the action of Gemex remained much the same, although the amount of growth produced varied proportionately according to the nitrogen source—most abundant with sodium ammonium phosphate and least with sodium nitrate. Throughout the series the *Streptomyces scabies* strains—Sc 10, Sc 5, Sc 4—in that order showed the most marked tendency towards complete or partial loss of aerial mycelium. The *Streptomyces coelicolor* A 3 (2) pigmented strains on the whole retained their characters better, but in any one plate variable aspects could generally be found. Such growths were more evident in nitrate nitrogen plates. The colourless strains 12 b/1–36 produced the most consistently uniform colonies. In the control series without Gemex good characteristic growth was obtained with all. The agar digesting A 3 (2) strain was the only one to grow equally well without sucrose.

The structure of aerial mycelium arising from different surfaces

(*a*) *From agar media as above.* Spiral sporophores, either tightly coiled (*Streptomyces coelicolor* A 3 (2): Pl. 1, fig. 4) or of varying degrees of looseness (*S. scabies* Sc 10: Pl. 1, fig. 1) were normally characteristic of the majority of the strains. Exceptions were the little more than wavy and often straight sporophores of the 12 b/1–36 isolates. No noteworthy differences were found between the urea and mineral salt media. The addition of Gemex, where it resulted in a partial development of aerial mycelium, also tended towards a straightening of the sporophores or sterility, or both. In those instances where the gross aspect seemed unchanged, as in the relatively good development of aerial mycelium by the isolates 12 b/1–36, a more detailed examination revealed considerable distortion of the sporogenous filaments. The most noticeable feature of this type of aerial growth was the thickening of many filaments, which were often swollen so as to form clavate tips.

(*b*) *From aqueous mineral salt or pectin media.* The clearest differences were observed in the 12 b/1–36 isolates, whose vigorous ability to produce aerial mycelium in most circumstances has already been noted. Sporophores typical of this group arising by simple monopodial branching from a filament of the vegetative mycelium produced relatively long chains of spores. When grown at the surface of a pectin + ammonium salt medium (Pl. 1, fig. 2), the whole filament and its short lateral branches were sporogenous and resembled the submerged sporing growths pictured by Carvajal (1946) and by Pénau, Hagemann, Velu & Peyre (1954) for *Streptomyces griseus*. It will be noted also that there were many swollen, large, and clearly empty cells. In many respects this type of sporulating growth showed considerable similarity to that produced on the detergent agar media (Pl. 1, fig. 5).

(*c*) *From cellophan over agar media.* Growth on cellophan is necessarily thinner and more evenly spread over the film as the result of the slow diffusion of nutrients. Hence it is easier to observe the progressive stages of development on such films than in the more compact cell complexes produced in direct contact with agar, or partially immersed in liquid media. The *Streptomyces coelicolor* A 3 (2) strains which required no carbohydrate added, when grown on cellophan over a mineral salt medium containing only nitrate, spread so evenly over the film (Pl. 1, fig. 3) that the regularity of both vegetative and sporogenous aerial filaments was clearly visible. The well-spaced growths on films over such simple media permitted observation of the way in which the aerial filaments arise by monopodial branching from any vegetative filament anywhere over the film. All the strains examined grew in this way, but with the addition of more nutrients to the agar the readier accessibility of the food supply allowed greater scope to the inherent tendency of actinomycetes to grow in a circumscribed fashion, i.e. to extend their cell-masses upwards by means of intertwined and branching filaments, instead of individually outwards in the manner of fungi. Thus with richer growths the origins of single aerial filaments could as a rule be discerned only at the margins. In no instances could any evidence be obtained of fusion of one or more filamentous cells to yield an 'initial cell' from which the secondary aerial growth was derived (Klieneberger-Nobel, 1947; McGregor, 1954).

Qualitative and quantitative differences in spore production

(*a*) *On agar media with and without Gemex.* The distortion of the sporogenous filaments sometimes produced on Gemex-containing media (Pl. 1, fig. 5) frequently resulted in a diminution in the spore yield. Comparison of impression preparations of the same strain (12b/36) grown on sucrose ammonium phosphate agar for the same length of time (2 weeks) with (Pl. 1, fig. 5), and without (Pl. 1, fig. 9) Gemex, will show this clearly. The total mass of cell material synthesized in the upper stratum of growth arising from detergent agar was at least as great as the normal growth in the control plate, but many of the swollen filaments failed to produce spores (Pl. 1, fig. 5). Such spores as were formed were usually in short chains, relatively few in numbers. The clavate terminal cells, as well as other irregularly shaped filamentous cells, were viable and germinated in the ordinary way under suitable circumstances. Nevertheless, the quantitatively higher yield of spores from a similar amount of filamentous growth in the control (Pl. 1, fig. 9) seemed a more economical use of the cell material from the point of view of distribution.

In the *Streptomyces coelicolor* species-group the typical pigmented A3 (2) strains were devoid of aerial growth on detergent media in most instances, and displayed a very regular development of spores throughout the spiral twists of the normal sporophores produced on ordinary media. In older colonies, however, long aerial strands in the form of interconnecting overhead bridges were often found to extend to adjacent colonies (Pl. 1, fig. 4), and from these might develop subsequent overgrowths of colourless more or less straight aerial strands. Such overgrowths, which probably obtained their nutrient syntrophically from the disintegrating filaments below, frequently showed inequalities in size on the part of the spores in long chains (cf. Pl. 1, fig. 9).

Sampling the spores by means of impression preparations clearly yielded information only concerning the superficial layer of growth, which was furthest removed from the source of food supply, and which might therefore be readily subject to slight variations. The random samplings taken from spore suspensions, especially those prepared from slopes in stoppered bottles, generally revealed a more uniform appearance (Pl. 1, fig. 8). It is of interest to compare these relatively large round spores produced in humid conditions with those which have fallen naturally from the crown of a colony to its perimeter on the thin film of drying agar in a slide culture (Pl. 1, fig. 6). The latter are slenderer, more oblong, and more varied in appearance, yet both specimens were from the same strain (12b/7 grown for the same time on the same medium (8 days on Czapek sucrose nitrate agar)). It was frequently found that differences in the depth and consistency of the agar, and especially in the degree of humidity obtaining in the culture vessel, resulted in variations both as to total numbers and aspect of the spores produced.

(*b*) *On liquid media.* In unshaken, unaerated cultures of simple aqueous Czapek media containing only dilute solutions of sugars or insoluble chitin or cellulose in the form of filter-paper strips, the thin network of surface filaments commonly gave rise to simple aerial sporophores typical of the respective strains. Shallow layers of such liquids in flasks or bottles or in Petri dishes supported by circles of filter-paper or cellophan permitted the development of more coherent superficial growth than in test-tubes, and small portions could readily be removed for microscopical examination without undue disturbance of the essential relationships of the growth. In such systems the yield of spores was determined by the area occupied by the medium and not by its volume; bottom growth was consistently vegetative only. Long chains of spores were developed in cultures of *Streptomyces scabies* and *S. coelicolor* (Pl. 1, fig. 7), but tended to fragment very readily. Spore size throughout was fairly constant, according to the species and strain. Spore chains were found, derived from cultures on both liquid (Pl. 1, fig. 7) and solid media, in which the constituent spores appeared to branch laterally. On closer examination these lateral spores were often found to arise from the isthmus between two spores in a chain (cf. Carvajal, 1946).

As already mentioned, the more plentiful but partially submerged growths in pectin media was usually characterized by an increase in the variety of shapes and sizes of spores together with a decrease in numbers. In the *Streptomyces scabies* Sc 4 strain growth was less distorted and the spores more uniform. On the whole, the most significant feature in the pectin growths was the frequent occurrence of thickened filaments, clubs and clavate terminal cells (Pl. 1, fig. 2). These structures resembled very closely the 'massues' and 'éléments spiciformes' described by Pénau *et al.* (1954) in their submerged cultures of *S. griseus*, and also on the pectin-coated coverslips partially immersed in a rich nutrient broth from whose examination they made their deductions concerning surface growth. The broth employed by these workers contained peptone, meat extract and glucose, and would therefore have a surface tension lower than that of watery solutions of mineral salts (see Glassman, 1948).

Germination of spores

(*a*) *In liquids.* Where there was no impediment to growth in any plane, e.g. in a tube of aqueous Czapek sucrose medium, spores rarely exhibited any change in shape prior to germination, and produced as a rule one short germ-tube which quickly branched in different directions (Pl. 2, fig. 10). The mycelium developing naturally in these circumstances soon became the typical streptomycete colony composed of branching filaments intertwining in every possible way, into whose interior visual inspection became increasingly difficult (Pl. 2, fig. 11).

(*b*) *On cover-slips receiving a fluctuating supply of liquid nutrients.* When a spore suspension in broth was run along the undersurface of a coverslip inclined at an angle over agar strips forming an enclosed chamber, spores adhering to the glass showed varying degrees of elongation (cf. Pl. 2, figs. 13–17) and might develop one to three germ-tubes. In such a system the supply of nutrients was locally restricted, with the result that 'scrolls' of elongated filaments following the movements of moisture on the glass were often formed (cf. similar development on cellophan, Pl. 2, fig. 12). Occasionally also, especially with the *Streptomyces scabies* strains which were characterized by a more wavy growth on the part of the vegetative filaments than *S. coelicolor* (cf. Pl. 2, fig. 12, with Pl. 1, fig. 3) spores germinating on the glass surface developed 'loops' or 'nests' of filaments (Klieneberger-Nobel, 1947; 'vrilles' and 'nids', Pénau *et al.* 1954). Such structures developed away from the glass as the growing filaments shifted direction slightly in accordance with the varying food supply. A photograph of such a 'loop' produced under identical conditions to those described has already been published for *Micromonospora vulgaris* (Erikson, 1953: pl. 1, fig. 6). Yet, as can be seen by the more recent illustrations of germinating spores of *M. vulgaris* (Erikson, 1955*a*: pl. 2, figs. 7–10; pl. 3, figs. 11, 16; pl. 4, figs. 17, 18), this particular thermophilic actinomycete normally produced long straight, or slightly wavy, loosely branching filaments on agar surfaces or in complete immersion in liquid cultures.

(*c*) *On moist cellophan films over agar media.* Germination on moistened membranes through which an adequate supply of nutrients diffused evenly appeared little different from the same process observed directly on a moistened agar surface (see Pl. 2, figs. 13–17). The most noteworthy feature of young growths on cellophan was the way in which the growing filaments extended for relatively long distances over the film, branching and developing during the early stages only within the plane parallel to the film (Pl. 2, fig. 12). If the spores were well spaced, it was then always possible to note the way in which advancing growing tips recoiled, crossed over or under, or extended in parallel strands with other filaments of the same or adjacent growths. Several clear instances of this lack of anastomosis can be seen in the present illustration (Pl. 2, fig. 12), as well as in that of a similar cellophan growth of the thermoduric strain *Streptomyces albus* 1569 accompanying another article (Erikson, 1955*b*: Pl. 2, fig. 14). All actinomycetes studied by these techniques have revealed the same manner of growth. As Pénau *et al.* (1954) have pointed out with regard to specimens of submerged growths of *S. griseus* artefacts can readily give rise to the appearance of hyphal fusion but when the living cells are examined by phase-contrast microscopy the enlarged 'fusion cell' is usually resolved into the projection of one filament crossing over another (see Pénau *et al.* 1954: pl. 1, fig. 7).

(*d*) *Between coverslip and agar surface.* When individual spores of *Streptomyces coelicolor* in slide cultures were examined and photographed at intervals, there was a more perceptible elongation of the germinating spore (Pl. 2, fig. 13: top right-hand spore) which might be partly attributable to the compression of the coverslip (cf. germinating spore in liquid, Pl. 2, fig. 10). It was also noted that a greater initial degree of elongation was apparent when a spore produced two germ-tubes (Pl. 2, fig. 14: same top right-hand spore 12 hr. later) in comparison with a spore producing first one germ-tube (Pl. 2, fig. 14: bottom left-hand spore) and then another after an interval (Pl. 2, fig. 15: the same bottom left-hand spore 18 hr. later). At this stage when both spores had produced two germ-tubes, it became obvious that the difference in size evident in Pl. 2, fig. 13, was a real distinction and not just a result of the earlier germination on the part of the one spore. A little later (Pl. 2, fig. 16: after 24 hr.) it was seen that the larger spore had synthesized a greater amount of cell material in the shape of two twisting filaments. At this stage burrowing into the agar had started and the complete growth was no longer in focus.

In another field of the same slide culture (Pl. 2, fig. 17: 3 days after sowing) four spores were found to have developed in slightly different ways. The one at the extreme right possessed three germ-tubes and showed a moderate degree of elongation, while a very similar appearance was given to the spore in the top left-hand as the result of the emergence in two germ-tubes. On the other hand, the one immediately beneath it was foreshortened as if the long axis—if indeed it were not almost spherical—had been turned downwards into the agar. This spore had only one germ-tube. Very little elongation, if any, was shown by the fourth spore to the left centre, which had a main short germ-tube from which two long branches had developed, as well as a second subsidiary germ-tube above the small sterile spore trapped between it and the main right-hand branch.

Throughout all the studies on germination it was found that living streptomycete spores appeared as homogeneous dense bodies when viewed by phase-contrast microscopy, whatever the methods of cultivation. Since they were not refractile like the spores of the thermophilic *Micromonospora vulgaris*, there could be no perceptible changes in density prior to germination. Those spores that occasionally appeared clear and empty, particularly in pectin or Gemex preparations, constantly proved non-viable. The density of the spores was such as to preclude the appearance during life of any internal structures.

DISCUSSION

The ability to produce an aerial mycelium, so characteristic a property of the genus *Streptomyces*, implies the capacity on the part of a large number of cells to pass beyond the surface boundary of the nutrient phase, whether solid or liquid, and then to obtain nourishment both for their own maintenance and for the synthesis of fresh cell material by means of an internal pipeline from the basal source of supply. This is a fundamentally different situation from that obtaining in the typical eubacterial culture where all but the superficial cells, and even they over at least half their area, can absorb individually through their external membranes the dissolved solutes requisite for growth. When this is borne in mind, it is logical to expect that local variations in the physical state of the substratum upon which a given streptomycete culture grows might readily affect the ability of certain cells to grow away from the medium/air interface. Moreover, even when such filamentous extension away from the nutrient source has taken place, slight differences in tension in the immediate environment of the basal cells may selectively alter or completely interrupt the transportation of soluble substances to the apical portion of the aerial growth.

It has been shown that the lowering of the surface tension of agar media by means of a non-toxic detergent resulted in inhibition or partial loss of aerial growth in most of the strains studied. The organisms which commonly produced the least aerial mycelium and rarely any at all on nutrient agar, such as the most virulent *Streptomyces scabies* strain Sc10, were the most susceptible to the inhibitory influence; while in the same group the least virulent strain Sc4 showed similar powers of aerial mycelium production on nutrient and detergent agars. On the other hand, the characteristic blue-pigmented A3 (2) strains of *S. coelicolor* produced normally both aerial sporogenous and vegetative phases on simple media containing inorganic nitrogen sources, but only the vegetative phase on rich complex media like nutrient agar. It is these strains which were most responsive to the inhibitory action of artificially lowered surface tension as regards aerial mycelium production in detergent media, whereas the colourless 12b strains were able to develop a moderate amount of aerial growth on both nutrient and detergent agars.

Correlation of paucity or complete suppression of aerial growth in both nutrient and detergent agars is of significant interest, since culture media containing protein ordinarily have surface tension values lower than that of water (Glassman, 1948). A careful scrutiny of the descriptions compiled by Waksman (1953) of a large number of known species of *Streptomyces* will reveal that in sixty-eight instances (46·5 %) absence or very scanty development of aerial mycelium on nutrient agar was reported as compared with moderate or good growth on a defined medium with a simpler nitrogenous source. In the large majority of these instances moderate to good, if not luxuriant, vegetative growth was produced, indicating that the organisms experienced little difficulty in metabolizing the complex media. It would therefore seem probable that it was the physical rather than the chemical components of the system that were active in preventing aerial growth. An analogous example can be observed in the potency of the physical factor of gas phase in connexion with the development of aerial mycelium by the thermophilic actinomycete *Micromonospora vulgaris*: aerial mycelium in atmospheric air but not in oxygen (Webley, 1954).

The distorted, swollen, and often empty cells found in the sporogenous secondary mycelium of those organisms which did produce aerial growth on detergent media resembled very closely the cells arising in abnormally short filaments from the surface and subsurface of pectin media. Again it would seem that the operative factor common to both systems is the physical one of surface tension. Both types of culture show examples of cytolysis such as often result from the contact of surface active agents with biological cells. Moreover, they show a very strong resemblance to the thickened rods, swollen buds, enlarged clavate tips and clubs described and illustrated by Pénau *et al.* (1954) for *Streptomyces griseus* when grown on pectin-coated coverslips in rich complex nutrient broth. The medium used by these authors would certainly have a surface tension value lower than that of water, even without the addition of dissolved pectin, while its richness would ensure the abundant production of susceptible cells.

Bloated shapes resulting from over-feeding and other unfavourable conditions are of common occurrence in many varieties of actinomycetes (cf. the large clubs and hexagonal cells of *Nocardia sebivorans* growing on the surface of solid paraffin: Erikson, 1955*b*, pl. 1, fig. 8). As Villemin, Lechevalier & Waksman (1953) have pointed out, such gross overfeeding as is normally involved in the cultivation of actinomycetes for antibiotic formation can be regarded as constituting 'pathologic' conditions in comparison with the austerity of the fare obtainable in the natural habitat—the soil. It is in such over-rich artificial environments, complicated in the case of submerged aerated cultures by the additional physical factor of vibration, that the variety of cell elements described by Pénau *et al.* (1954) has been found. It must also be remembered that *Streptomyces griseus*, the organism with which they worked, is extremely variable even for a streptomycete (see Waksman, Reilly & Harris, 1948).

No clear cases of anastomosis were found by Pénau *et al.* (1954), confirming the observations of Erikson (1949). This is of importance genetically, if we accept Whitehead's (1948) thesis that it is more important for a proposition to be interesting than to be true, for the most interesting theory concerning the origin of the aerial mycelium depends on the assumption of hyphal fusion. Klieneberger-Nobel (1947) did not observe any clear cases of fusion, and merely suggested the possibility of such occurrences within the tangled 'nests' of filaments to be found in partially immersed coverslip growths. A criticism of this technique and of the physical factors involved in such growths has already been made (see earlier section: Germination of Spores (*b*)). Examination of well-spaced growths on poor nutrient media (see Pl. 1, fig. 3) will constantly reveal simple monopodial branching on the part of the aerial filaments, without the intervention of any enlarged 'initial cell', just as it has been described by all the earlier workers (see Ørskov, 1923). Moreover, wild streptomycetes in soil show the same characteristic mode of growth when developing on buried coverslips (see Erikson, 1947: pl. 1, fig. 2). Yet Bisset (1950) has raised Klieneberger-Nobel's theory to the status of dogma without the benefit of further confirmatory evidence or reference to earlier contradictory data. Some of the drawbacks to the methods employed by Bisset in elucidating cytological findings have been pointed out by DeLamater (1954).

The extreme plasticity of the actinomycetes in general in response to environmental conditions is well known to those who have long occupied themselves with these organisms, but is perhaps less vividly realized by those mainly preoccupied with eubacteria. It is generally the latter workers who have attempted to find significance in unusually shaped cells produced in special circumstances. On the other hand, Gottlieb (1953) has stressed the heterogeneity of streptomycete cultures as a result of observing that growth is confined almost entirely to the apical region of the hypha, in this respect resembling the fungi. In my experience it is this fact—that in mature growths in complex media large portions of the vegetative mycelium are dead or dying, although still permitting the passage of nutrients to the living cells, that accounts to a large extent for the delimitation of the vital protoplasm within thickened filaments, enlarged 'clubs', 'éléments spiciformes', 'segmentation spores', etc. Such structures may persist through adverse circumstances and germinate to form a typical mycelium in suitable environments, but they cannot therefore be regarded as reproductive organs.

In the present study different strains of *Streptomyces scabies* reacted differently to surface tension factors in producing little or no aerial mycelium on detergent agars, and showed varying degrees of susceptibility to other changes of the physical environment. The pigmented A3 (2) and the colourless 12b variants of *S. coelicolor* also responded in varying ways. These two forms were originally obtained from single spores. On the other hand, the various isolates of A3 (2) which were obtained as the result of micromanipulative dissection of a very young homogeneous vegetative mycelium behaved throughout very similarly. This was the case also with the 12b isolates of similar origin. Such results uphold the conclusions drawn from earlier work carried out in less detail (Erikson, 1948).

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EXPLANATION OF PLATES

Phase-contrast preparations.

PLATE 1

Fig. 1. *S. scabies*, Sc 10, 8 days on sucrose urea agar, showing loosely coiled spiral sporophores. (×720.)

Fig. 2. *S. coelicolor*, 12b/10, 24 days at surface of pectin ammonium salt liquid medium, showing sporogenous filament with short lateral branches and many of the cells abnormally large or empty. (×2640.)

Fig. 3. *S. coelicolor*, A3 (2), 4 days on cellophan over Czapek nitrate agar without added carbon source, showing loosely coiled aerial filaments arising by monopodial branching from the vegetative mycelium. (×720.)

Fig. 4. *S. coelicolor*, A3 (2), 4 weeks on sucrose ammonium salt agar, showing sporophores from adjacent colonies intertwining by means of their tightly coiled spirals. (×720.)

Fig. 5. *S. coelicolor*, 12b/36, 3 weeks on sucrose ammonium salt Gemex agar. Impression preparation showing distorted sporogenous filaments. (×2160.)

Fig. 6. *S. coelicolor*, 12b/7, 8 days on sucrose nitrate agar slide culture. Slender, elongated spores fallen from crown of colony to perimeter; cf. Pl. 1, fig. 8. (×2160.)

Fig. 7. *S. scabies*, Sc4, 8 days at surface of chitin mineral salt medium, showing chain of spores with apparent lateral branching. (×2640.)

Fig. 8. *S. coelicolor*, 12b/7, 8 days on sucrose nitrate agar slope in stoppered bottle. Aqueous spore suspension from growth. (×2160.)

Fig. 9. *S. coelicolor*, 12b/36, 2 weeks on sucrose ammonium salt agar. Impression preparation showing long mainly straight chains of spores, with some spores larger than the normal size; cf. fig. 5. (×2160.)

PLATE 2

Fig. 10. *S. scabies*, Sc4, 48 hr. in sucrose Czapek liquid medium, showing germinating spore with very short germ tube giving rise to three branches. (×2640.)

Fig. 11. *S. scabies*, same as fig. 10, 4 days later, showing portion of closely intertwined mycelial growth. (×800.)

Fig. 12. *S. scabies*, Sc5, 4 days on cellophan over sucrose ammonium salt agar, showing manner in which advancing filaments cross over one another or extend in parallel strands. (×640.)

Fig. 13. *S. coelicolor*, A3 (2), portion of slide culture on nutrient agar sealed with coverslip, showing two spores of different sizes prior to germination. (×4320.)

Fig. 14. Same as fig. 13, 12 hr. later, showing two germ tubes produced by elongated top right-hand spore, and only one germ tube by the smaller spore. (×4320.)

Fig. 15. Same as fig. 13, 18 hr. later. The smaller spore has now produced a second germ tube. (×4320.)

Fig. 16. Same as fig. 13, 24 hr. later, showing twisting of young filaments burrowing into agar with the result that growth is partly out of focus. (×4320.)

Fig. 17. Another field of the same slide culture as in fig. 13, 3 days after sowing, showing four germinated spores and one sterile. The number of germ tubes produced varies between 1 and 3. (×2800.)

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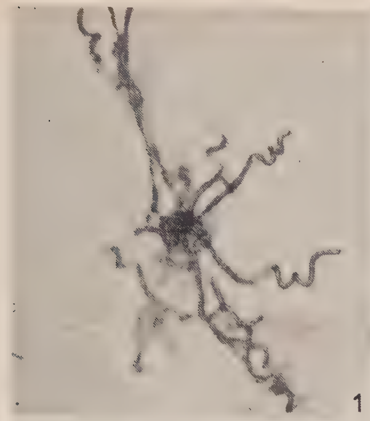
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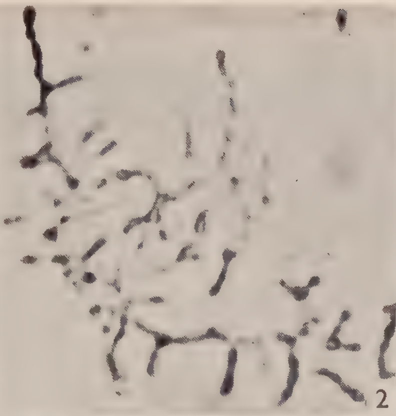
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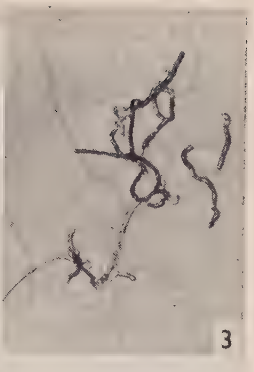
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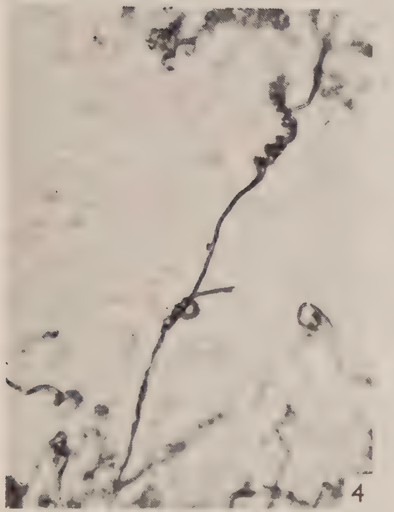
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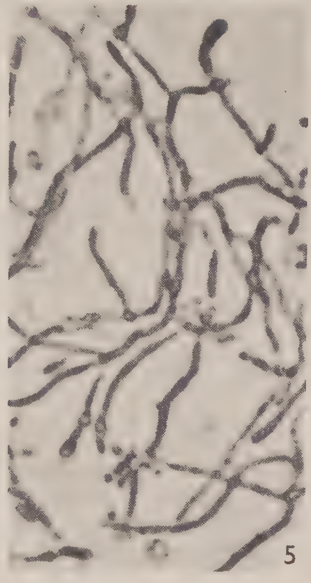
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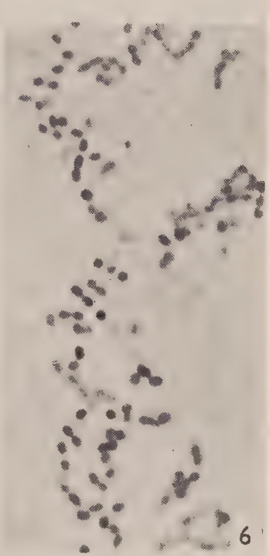
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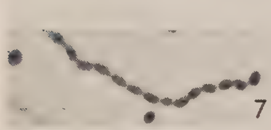
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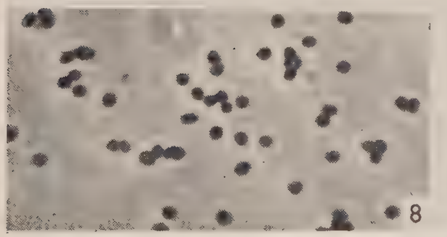
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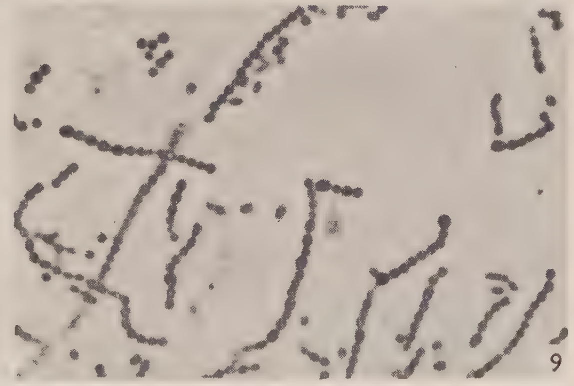
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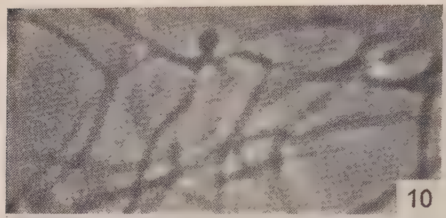
D. ERIKSON—PHYSICAL FACTORS AFFECTING STREPTOMYCETES. PLATE 1

(*Facing p.* 148)



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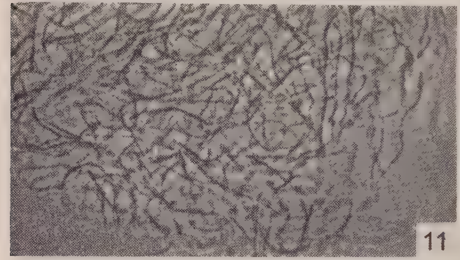

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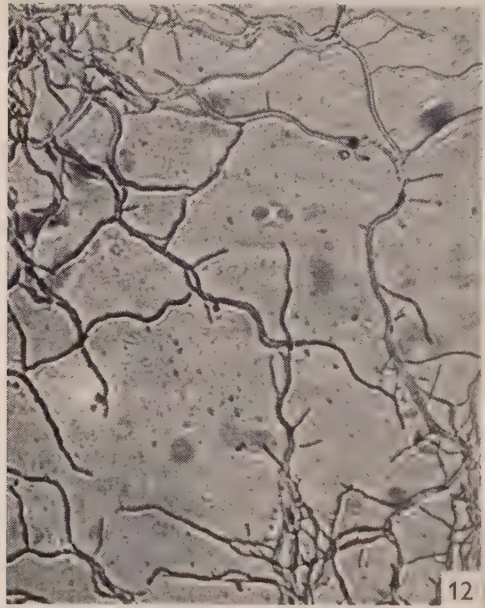

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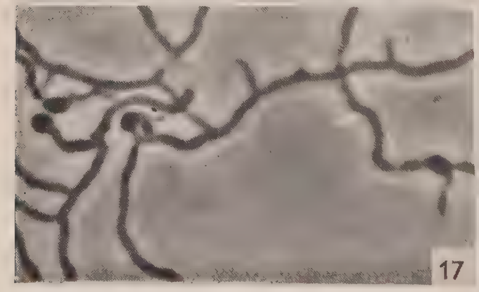

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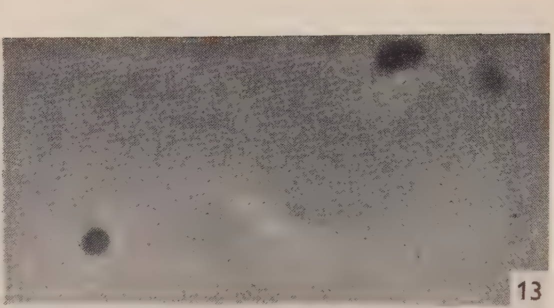

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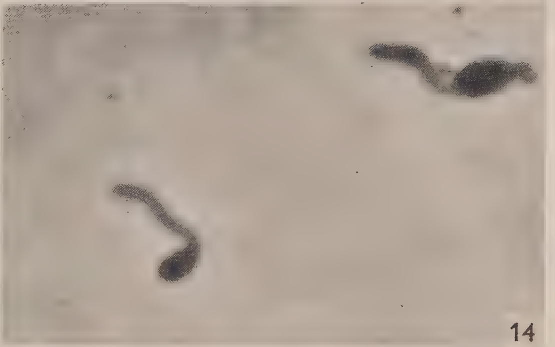

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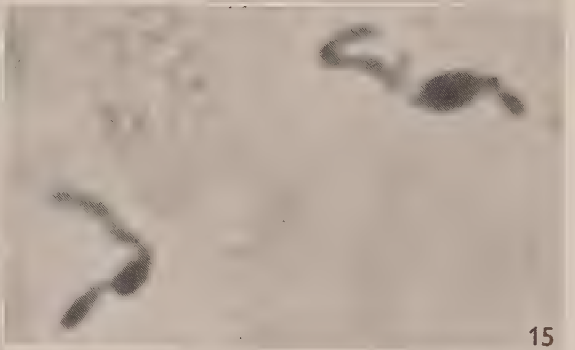

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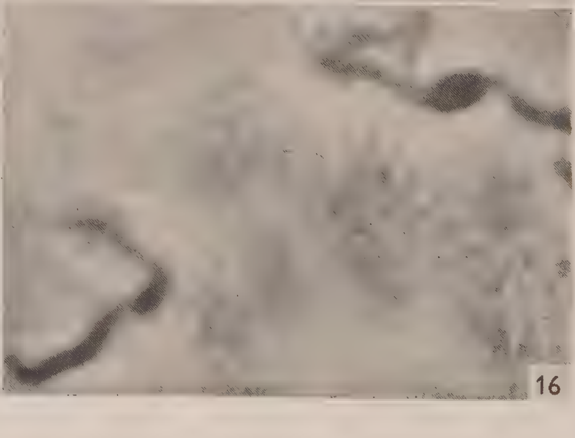

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D. ERIKSON—PHYSICAL FACTORS AFFECTING STREPTOMYCETES. PLATE 2

Cuckow, F. W. & Klieneberger-Nobel, E. (1955). *J. gen. Microbiol.* **13**, 149–154.

Further Studies of Organisms of the Pleuropneumonia Group by Electron Microscopy

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SUMMARY: The growth on solid medium of organisms of the pleuropneumonia group as seen in electron micrographs was compared with the growth in liquid media described in a previous paper (Klieneberger-Nobel & Cuckow, 1955). There is no evidence of a rigid cell wall in these organisms, a finding consistent with those electron micrographs of growth in liquid media and in light microscopical preparations. The newly formed minimal reproductive units, hitherto never demonstrated in the light microscope except when they have grown to a larger size, are here demonstrated in large numbers as a uniform phase of small, delicate elements, the diameter of which measures *c.* 100 mμ. in the electron micrographs.

In a previous paper (Klieneberger-Nobel & Cuckow, 1955) we demonstrated the electron micrographic appearances of some organisms of the pleuropneumonia group grown in liquid media. The present study is mainly concerned with the appearance of these organisms when grown on solid media.

MATERIALS AND METHODS

The strains used were: the organisms of agalactia and of pleuropneumonia of goats of our previous study: an organism of pleuropneumonia of cattle sent from Africa; an organism designated 'Cheng' (Klieneberger-Nobel & Cheng; to be published) isolated from a lung abscess of a bronchiectatic rat; an organism from sewage isolated by Laidlaw & Elford (1936), designated 'Laidlaw A'.

The methods employed with organisms grown in liquid medium were slightly altered to suit growth on solid medium. A 'formvar' solution in chloroform was poured over a slice of appropriate agar medium resting on a sterile slide, thus producing a membrane on its surface. The organisms were freshly grown in liquid medium, spun down and washed once in Tyrode–Ringer solution. The sediment was resuspended in Tyrode–Ringer solution and diluted to an opacity which varied with the strain, but which was usually just appreciable by the naked eye. With a fine capillary pipette droplets of this suspension were placed on the formvar membrane. Each preparation was incubated in a Petri dish, which for long incubation was kept in a moist chamber. The water of the droplet gradually evaporated leaving a round, distinct, sometimes slightly opaque area. Drying was sometimes speeded up by a pre-drying of the membrane-covered agar-slices in the incubator. After the desired period of incubation the formvar membranes were floated on Tyrode–Ringer solution for 15–30 min., then floated on 40 % formalin, for 3 min., and then on distilled water for 1 hr. The parts of the membranes which carried the droplets were placed on grids, dried, shadowed and photographed under the electron microscope at 60 kV. Uninoculated control preparations showed nothing significant.

Description of the electron micrographs

Plate 1, fig. 1, shows the 'Laidlaw A' organism grown for 18 hr. at room temperature. Some of the resulting delicately grown patches contain roundish opaque bodies which are similar to those often seen in liquid media. However, they differ in that their edges are thinner, more irregular and hazy, as though they had begun to spread. The edges of adjacent forms are connected with each other so that the boundaries of single organisms are no longer distinguishable in some of the grape-like agglomerations.

Plate 1, fig. 2, shows the organism of pleuropneumonia of cattle grown for 18 hr. at room temperature. The growth is only scanty and delicate. Some of the organisms are no larger than those from liquid media, but their edges are less definite. Others appear to have enlarged without subdividing, and to have become thinner and more transparent to the electron beam. The larger bodies vary in transparency.

Plate 1, fig. 3, shows the goat pleuropneumonia organism, a quickly growing strain. After 4½ hr. at 37° there was not a single organism which resembled the forms found in growths from serum broth. All the organisms here are spread out on the membrane and are extremely thin; the organisms at the edge of the colony are particularly thin in their outermost parts. This appearance is common in stained preparations of growths on solid medium and is also observed in living specimens under the 'ultropak' microscope. The area in the print shown has a superficial resemblance to a tissue section, an appearance often seen in stained and in phase-contrast preparations of colonies of pleuropneumonia-like organisms.

Plate 1, fig. 4, shows the edge of a colony of the sewage organism 'Laidlaw A'. This organism grows even more rapidly than the goat pleuropneumonia organism. The culture was grown on a pre-dried membrane for 5 hr. at 37° and then 18 hr. at room temperature in order to obtain a more advanced growth than in the specimens so far described. A group of fairly large forms is seen surrounded by minimal reproductive units.

Plate 1, fig. 5, shows growth from a 2-day serum broth culture of the organism of agalactia. This picture is included to show that discrete minimal reproductive units occur in liquid media, a fact which we failed to illustrate satisfactorily in previous work. The minimal reproductive units seen here are of the same size as those grown on the solid medium. All these units are fairly uniform and have a diameter of *c.* 100 mμ. in the electron micrographs.

Plate 1, fig. 6, shows the strain 'Cheng' grown for 18 hr. at 37°. A large number of minimal reproductive units, singly and in small groups, are scattered over the field. There are two larger forms with irregular edges which may represent 'mother' forms from which the minimal reproductive forms develop.

Plate 2, fig. 7, shows the sewage organism 'Laidlaw A'. There are minimal reproductive units as well as some very large flat forms, of a kind which frequently occur on the surface of solid media.

Plate 2, fig. 8, shows the sewage organism 'Laidlaw A'; the appearances are consistent with the production of minimal reproductive units by sprouting.

Plate 2, fig. 9, shows a micro-colony of the 'Laidlaw A' organism. In various places round the edge the large organisms seem to have been replaced by collections of minimal reproductive units lying in a continuous matrix. In a few of the large organisms granules of about the size of the minimal reproductive units can be seen.

Plate 2, fig. 10, shows some of the organisms of agalactia from solid medium with a peripheral concentration of material, a feature so characteristic for the much smaller organisms which occur in liquid media. These thickenings appear particularly when cultivation on solid medium takes place in a moist atmosphere. In stained preparations these peripheral thickenings may easily be interpreted, and often have been so interpreted, as cell walls. However, in the electron micrograph shown here it seems to be only a concentration of material, often unevenly distributed. It may indeed be material from which minimal reproductive units are produced.

DISCUSSION

The variety of electron micrographic appearances among pleuropneumonia-like organisms grown in liquid media and organisms grown on solid media is great. In liquid media small spheres, oval bodies, disks and filaments occur exclusively; on solid media the forms have little depth, but are spread widely over the membrane in thin sheets or in leaf-like and sometimes amoeboid forms. This diversity, dependent on external conditions, is evident in the light microscope, but the electron micrographic evidence is even more striking. From consideration of stained and darkfield preparations and the results of filtration analysis, most workers agree that the organisms of the pleuropneumonia group lack a definite cell wall, and that they produce during their growth small corpuscles, the minimal reproductive units, which can pass fine filters and reproduce.

The electron micrographs of specimens from liquid and solid media reveal no rigid cell walls. Moreover, the thin hazy edge of the forms as seen in many of the electron micrographs here (for example, Pl. 1, fig. 1), the apparent lack of boundaries between neighbouring forms (Pl. 1, fig. 3, and Pl. 2, fig. 9), and the occurrence of material links between neighbouring forms (Pl. 2, fig. 10) bear out the view of the non-existence of a rigid cell wall.

However, one particular appearance of forms in stained preparations has sometimes been misinterpreted. A ring of darkly staining material may appear at the outer boundary of various forms. In older specimens darkly staining 'granules' appear within this ring which, as noted above, has been taken for a cell wall. From the electron micrographs (e.g. Pl. 2, fig. 10) it can be seen that an irregular rim of opaque material is often produced round some forms or round parts of some forms, which may give the impression of a cell wall in stained specimens. Yet the electron microscope shows that there is no cell wall, but a rim of concentrated material which corresponds to the material concentrated round the edges of the much smaller forms grown in serum broth, which, as shown previously (Klieneberger-Nobel & Cuckow, 1955) break up into minimal reproductive units. The electron microscope thus provides new evidence of the absence of a rigid cell wall in the pleuropneumonia group of organisms.

Minimal reproductive units occur in liquid as well as on solid media. Elford's (1929) filtration analysis of the organisms of pleuropneumonia of cattle and agalactia of sheep and goats proved that a large number of elements in these cultures can pass through fine Gradocol membranes down to the end-point of 0·25 μ. average pore diameter; some even pass through slightly finer membranes. According to Elford the diameter of the small units lies between 125 and 175 mμ.; according to our own unpublished experiments (E. K.-N.) their diameter is 100–150 mμ. The minimal reproductive units can be seen in Pl. 1, figs. 4–6 and Pl. 2, figs. 7–9. It appears from these pictures that they are about 100 mμ. in diameter and represent a phase of nearly uniform size and appearance. They may be slightly larger in the living state because the electron micrographs have been prepared from dried fixed material. Their diameters as estimated from both the electron micrographs and the filtration experiments are in good agreement.

Very few workers have demonstrated small units in stained preparations or by dark-ground microscopy. Turner (1935) showed some 'granules' in his excellent darkground pictures, and similar granules appear in ultropak and stained preparation pictures (Klieneberger & Smiles, 1942). They are also occasionally visible in the pictures of Dienes (1945; pl. 3, figs. 1, 2). The granules in the photographs of Klieneberger & Smiles were fewer in number, further apart and stand out more brilliantly than the minimal reproductive units in the electron micrographs. The latter are too small and too delicate to be visible in the light microscope, though they would be visible after an increase in size and opacity (cf. some of the units in Pl. 1, figs. 4, 5; Pl. 2, fig. 7). It follows that the 'minimal' reproductive units seen in the light microscope are no longer minimal but have grown into larger organisms.

The interpretation we offer of our electron micrographs is as follows. There is a phase of small corpuscles, the minimal reproductive units, which on the surface of solid media grow into larger flat organisms which have no rigid cell walls, but are often characterized by a soft edge. The thickness of these flat forms is of the same order as the diameter of the minimal reproductive units. The units appear to form in the substance of the flat organisms, which then degenerate to leave a single layer of discrete minimal reproductive units on the surface of the medium. In some cases the minimal reproductive units appear to arise from the end of prolongations that sprout from the edge of the large forms. Though the appearances of organisms grown in liquid and on solid media differ widely we consider that there is no fundamental difference of development. In the liquid medium the forms are small and produce few minimal reproductive units, while on solid medium they are large and produce a correspondingly large number of minimal reproductive units.

Electron micrographs of organisms of the pleuropneumonia group have been published by Smith, Hillier & Mudd (1948), Freundt (1954), Dienes (1953) and Liebermeister (1953). The methods used by these authors do not appear to be the best adapted for the demonstration of these organisms under the electron microscope, because the results, though confirming what was already known from studies by light microscopical methods, did not bring to light new information in spite of the greater resolving power available. The electron micrographs illustrated here and in our previous paper now show, however, that the pleuropneumonia group of organisms can be studied to advantage under the electron microscope.

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EXPLANATION OF PLATES

(Magnification: × 12,000 throughout.)

Plate 1

Fig. 1. Sewage organisms 'Laidlaw A' grown on solid medium at room temperature for 18 hr.

Fig. 2. The organism of pleuropneumonia of cattle grown on solid medium at room temperature for 18 hr.

Fig. 3. The organism of pleuropneumonia of goats grown on solid medium at 37° for 4½ hr.

Fig. 4. Sewage organism 'Laidlaw A' grown on solid medium at 37° for 5 hr. and at room temperature for an additional 18 hr.

Fig. 5. The organism of agalactia of goats grown in liquid medium at 37° for 2 days.

Fig. 6. The organism causing bronchopneumonia in rats grown on solid medium at 37° for 18 hr.

PLATE 2

Fig. 7. Sewage organism 'Laidlaw A' grown on solid medium at 37° for 5 hr. and at room temperature for an additional 18 hr.

Fig. 8. Sewage organisms 'Laidlaw A' grown on solid medium at room temperature for 18 hr.

Fig. 9. Sewage organism 'Laidlaw A' grown on solid medium at 37° for 5 hr. and at room temperature for an additional 18 hr.

Fig. 10. The organism of agalactia of goats grown on solid medium in moist chamber at 37° for 24 hr.

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ADDENDUM

After this paper had been submitted we saw the paper of H. E. Morton, J. G. Lecce, J. J. Oskay and N. H. Coy (*J. Bact.* 68, 697, 1954). Their electron micrographs of pleuropneumonia-like organisms from man and chickens show appearances comparable to ours.

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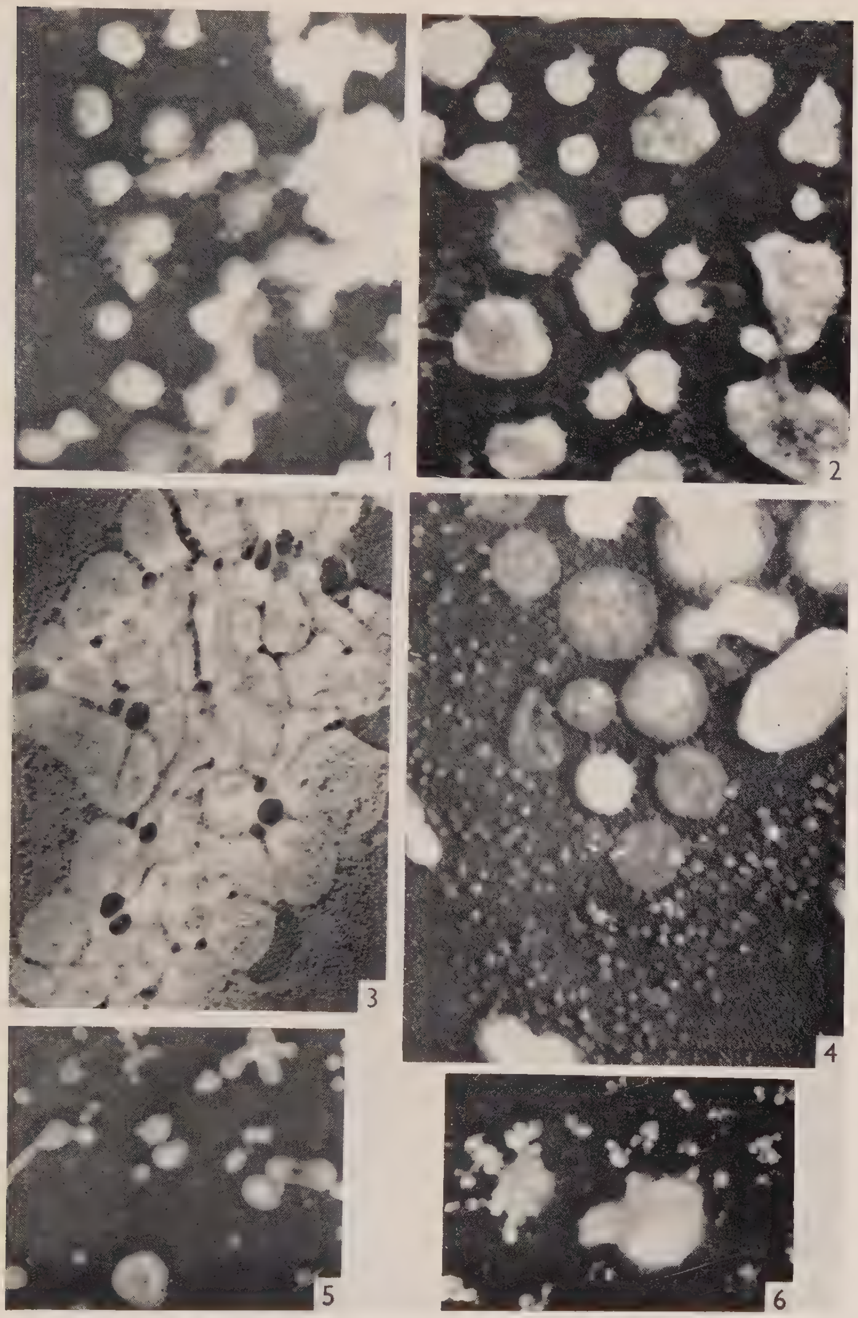
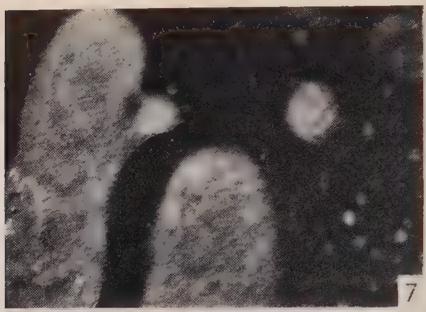


F. W. Cuckow and E. Klieneberger-Nobel—Further studies of the pleuropneumonia group. Plate 1

(*Facing p.* 154)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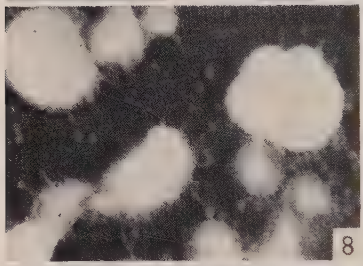
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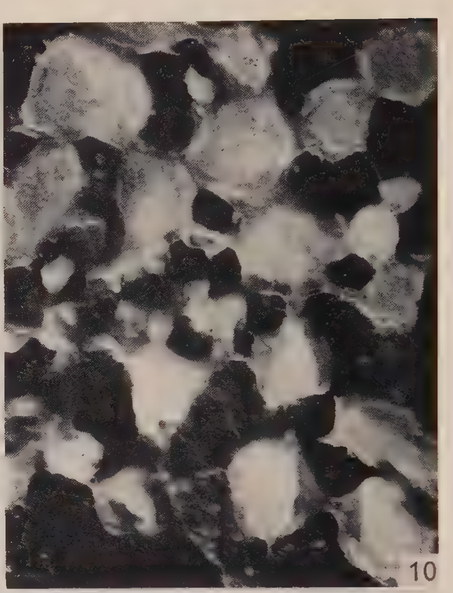
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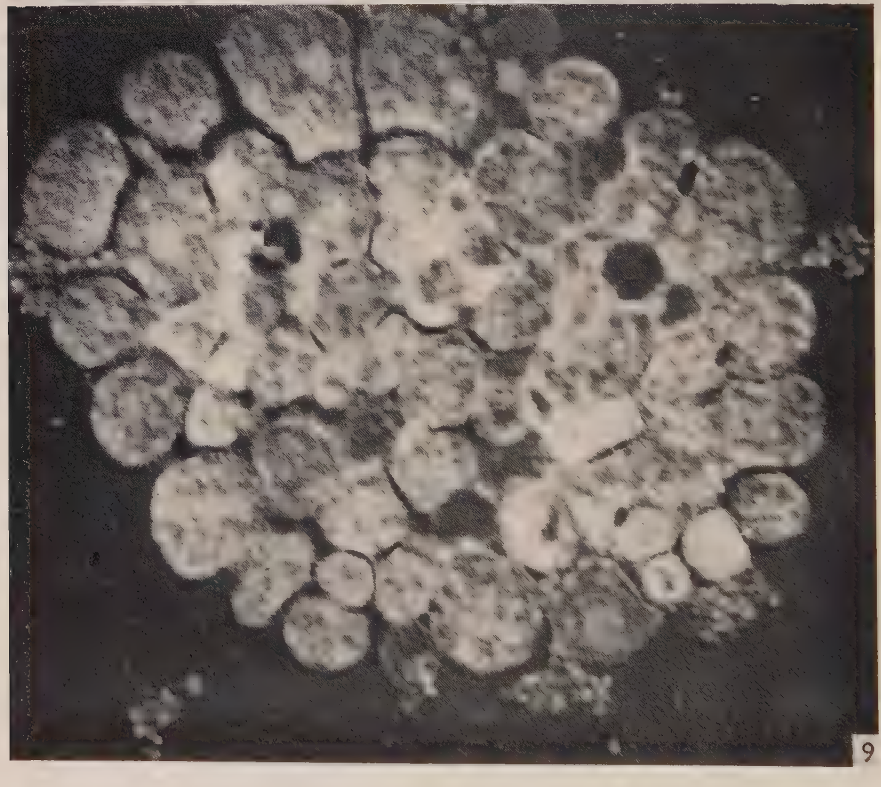
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F. W. CUCKOW AND E. KLIENEBERGER-NOBEL—FURTHER STUDIES OF THE PLEUROPNEUMONIA GROUP. PLATE 2

Shaw, C. & Clarke, P. H. (1955). *J. gen. Microbiol.* 13, 155–161.

Biochemical Classification of Proteus and Providence Cultures

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SUMMARY: Biochemical reactions of 153 Providence (29911 type) and 63 Proteus cultures were examined. Providence cultures are included in the genus *Proteus*, as their cultural characters are similar; this relationship is strengthened by the production of L-amino acid oxidase and glutamic acid decarboxylase by both. *Proteus inconstans* Ornstein nov.comb. is the name proposed for Providence cultures.

The family Enterobacteriaceae includes many groups which have a position intermediate between the established genera; of these the paracolon 29911 type (Stuart, Wheeler, Rustigian & Zimmerman, 1943) or Providence group (Brooke, 1951; Kauffmann, 1951) were found by biochemical tests to be related to the genus *Proteus* (Ewing, Tanner & Dennard, 1954). We have investigated the distribution of several biochemical characters among the Enterobacteriaceae, in particular the ability to metabolize potassium gluconate, sodium malonate, and also to attack amino acids by oxidative deamination or decarboxylation. The results we have obtained confirm the close relation of the Providence cultures to the species of the genus *Proteus*.

METHODS

Strains. The cultures of *Proteus* spp. and other genera examined were those maintained at the National Collection of Type Cultures. The strains examined included: *Bacillus inconstans* (Ornstein, 1921), an intestinal organism described and deposited by Braun in 1926; the three original strains of the Wakefield bacillus (Berger, 1945); B 81 and B 105 of Sachs's mannitol-negative dysentery organisms (Sachs, 1943); Stuart's paracolon 29911 type. Later, we obtained 120 Providence cultures from Dr W. H. Ewing (these are listed as Providence (2) in Table 2), and freshly isolated strains from faeces collected by different laboratories of the Public Health Laboratory Service during investigations of diarrhoea.

Biochemical tests

Gluconate test. The medium was a modification of that used by Haynes (1951) for pseudomonads. It consisted of: Evans's peptone, 1·5 g.; Yeastrel, 1·0 g.; K_2HPO_4, 1·0 g.; potassium gluconate, 40·0 g.; distilled water, 1000 ml.; adjusted to pH 7·0; sterilized 10 lb./sq.in. for 10 min. After incubation of the culture for 48 hr. at 37°, 1·0 ml. of Benedict's qualitative reagent for reducing sugars was added and the tubes placed in boiling water for 10 min.

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A positive test showed a yellowish brown precipitate of cuprous oxide and there was a characteristic smell of decaying cabbage.

Phenylalanine and malonate tests. Leifson's (1933) sodium malonate and Henriksen's (1950) phenylalanine tests were combined by adding phenylalanine to Leifson's medium: the combined medium was supplemented with 0·1 % Yeastrel to increase growth. The medium was as follows: $(NH_4)_2SO_4$, 2·0 g.; K_2HPO_4, 0·6 g.; KH_2PO_4, 0·4 g.; NaCl, 2·0 g.; sodium malonate, 3·0 g.; DL-phenylalanine, 2·0 g.; Yeastrel, 1·0 g.; 0·5 % (w/v) ethanolic bromthymol blue, 5 ml.; distilled water, 1000 ml.; sterilized 10 lb./sq.in. for 10 min. Cultures were incubated overnight at 37° and organisms using malonate produced an alkaline reaction. When a few drops of 0·1 N-HCl and a few drops of 0·5 M-ferric chloride were added to the tubes, a deep green colour indicated the production of phenylpyruvic acid from phenylalanine (Henriksen & Closs, 1938).

Indole and H_2S *production* were shown, respectively, by oxalic acid papers (Holman & Gonzales, 1923) and lead acetate papers inserted above cultures in Lemco broth + 0·01 % cysteine incubated at 37° for up to 7 days (Clarke, 1953).

Gelatin liquefaction was observed in nutrient gelatin stab cultures incubated for 30 days at 22°.

Hydrolysis of urea. Stuart, van Stratum & Rustigian's (1945) highly buffered medium and also Christensen's (1946) weakly buffered nutrient urea agar were used. The tubes incubated at 37° were read daily for 7 days.

Methyl-red test (MR). Cultures were grown in glucose phosphate peptone (Evans) medium and incubated for 5 days at 30°.

Voges-Proskauer test (VP). Three media, each containing Evans's peptone, were used. Glucose phosphate peptone medium; glucose peptone medium with NaCl but without phosphate (Smith, Gordon & Clark, 1946); and glucose peptone medium without salt and phosphate (Abd-el-Malek & Gibson, 1948). Cultures were incubated at 30° for 5 days and tested for the presence of acetoin by Batty-Smith's (1941) modification of Barritt's (1936) α-naphthol method.

Use of citrate as sole source of carbon. All tests were made by needle inoculation from a liquid culture. Koser's citrate medium (1923) was incubated for 7 days at 30°. All tubes showing turbidity were regarded as positive when confirmed by needle reinoculation into citrate.

Fermentation tests. Medium consisted of peptone water + bromcresol purple + 1 % (w/v) of 'sugar' and final readings made after incubation of cultures for 14 days at 37°. The 'sugars', tested were glucose, arabinose, xylose, rhamnose, lactose, sucrose, maltose, trehalose, glycerol, mannitol, dulcitol, sorbitol, inositol, adonitol and salicin.

Microtests

In most cases tests were carried out simultaneously on growing cultures and as microtests with washed suspensions (Clarke & Cowan, 1952). Heavy suspensions were made from cultures grown overnight on Lemco agar at 37°. The microtests were carried out as follows:

Phenylalanine. Two drops suspension, 2 drops 0·03 M-phenylalanine, 2 drops 0·025 M-phosphate buffer, pH 6·8. Incubated in a water-bath and after 1 hr. at 37° tested by adding 2 drops 0·5 M-$FeCl_3$. Other amino acids were also used as substrates.

Malonate. Two drops suspension, 2 drops 0·03 M-sodium malonate, 2 drops 0·025 M-phosphate buffer, pH 6·0 + phenol red as indicator. Results were read up to 24 hr. at 37° and positive tests showed an alkaline reaction.

Decarboxylases. Two drops suspension, 2 drops 0·03 M solution amino acid adjusted to pH 5·0, 2 drops 0·0125 M-phthalate buffer, pH 5·0 + bromcresol purple. At this pH value CO_2 is liberated so that the reaction mixture will show an indicator colour change when decarboxylation has taken place. Readings were taken at 2, 4 and 24 hr. at 37°. Toluene was added in later tests. Each set of tests included a suspension control without amino acid; when this suspension was alkaline buffer was added to all tubes until a yellow colour was given by the control.

RESULTS

Table 1 summarizes the results of the tests made on the *Proteus* spp. and the Providence strains. Included among the Providence strains are Sachs's B 81 and B 105 and the Wakefield bacillus, which Stuart, Wheeler & McGann (1946) showed to be members of the Providence group. NCTC 2481, *Bacillus inconstans* Ornstein, in our routine examination was found to have the biochemical characters of the Providence group. Dr W. H. Ewing confirmed this and has also shown that serologically the strain belongs to Providence 0 group 9.

Motile Providence strains, like *Proteus* spp., have a tendency to spread on agar. Therefore very dry plates were used to obtain single colonies. The cells were found to be peritrichously flagellate, though the flagella were few in number as compared with typical *Proteus* spp.

All strains of *Proteus* spp. and all but two of the Providence group produced phenylpyruvic acid by oxidative deamination of phenylalanine. The two negative cultures were positive by the micromethod, but had lower than average activity. There was no clear-cut division between any of the *Proteus* spp. and the Providence group when histidine was used as substrate (Singer, 1953). Stumpf & Green (1944) found that freshly prepared suspensions of *P. vulgaris* could oxidatively deaminate nearly all the naturally occurring amino acids, and manometric studies on the Providence cultures show that there is considerable variation in the relative rates of oxidation of phenylalanine and histidine by different strains. The phenylalanine microtest was particularly sensitive; after 15 min. incubation *Proteus* spp. and Providence cultures produced a deep green colour on the addition of ferric chloride. Other species of enterobacteria were negative (Table 2), but after 24 hr. incubation weak reactions were occasionally obtained with strains of *Klebsiella aerogenes*, *Escherichia coli* and some paracolons, so that the enzyme is not completely absent from these species.

Gluconate oxidation, originally used by Haynes to distinguish the pseudomonads, is a useful test for klebsiellas. Providence and *Proteus rettgeri* strains do not oxidize gluconate, but a few strains of the other *Proteus* spp. give positive results in this test. The malonate test is invariably negative with *Proteus* and Providence cultures.

Table 1. *Cultural characters of Proteus and Providence strains*

	P. mirabilis		*P. morganii*		*P. rettgeri*		*P. vulgaris*		Providence strains	
Biochemical tests	+	−	+	−	+	−	+	−	+	−
Motility	22	1	22	2	8	1	2	5	144	9
Gluconate test	13	10	1	23	0	9	0	7	0	153
Malonate test	0	23	0	24	0	9	0	7	0	153
Phenylalanine deamination	23	0	24	0	9	0	7	0	151	2
Indole	0	23	24	0	8	1	7	0	151	2
H_2S produced	22	1	24	0	9	0	7	0	153	0
MR	8	15	8	16	7	2	5	2	101	52
VP	17	6	0	24	0	9	1	6	1	152
Gelatin liquefied	18	5	0	24	0	9	4	3	0	153
Urea hydrolysed	23	0	24	0	9	0	7	0	11	142
Citrate utilized	6	17	1	23	8	1	4	3	96	57
Acid from										
Glucose	23	0	24	0	9	0	7	0	153	0
Arabinose	1	22	0	24	3	6	0	7	5	148
Xylose	23	0	5	19	0	9	6	1	17	136
Rhamnose	0	23	0	24	7	2	0	7	5	148
Lactose	0	23	0	24	0	9	0	7	2	151
Sucrose	19	4	1	23	9	0	7	0	146	7
Maltose	1	22	0	24	0	9	7	0	9	144
Trehalose	23	0	3	21	0	9	5	2	32	121
Glycerol	22	1	20	4	8	1	7	0	80	73
Mannitol	0	23	0	24	9	0	0	7	17	136
Dulcitol	0	23	0	24	0	9	0	7	3	150
Sorbitol	0	23	0	24	0	9	0	7	12	141
Inositol	0	23	0	24	8	1	0	7	32	121
Adonitol	0	23	0	24	9	0	0	7	110	43
Salicin	4	19	0	24	5	4	5	2	9	144

Table 2. *Microtests for Proteus and Providence strains*

		Amino acid decarboxylase			Deaminase
		Arginine	Lysine	Histidine	Phenylalanine
	No. tested	No. positive			
P. vulgaris	6	0	0	0	6
P. mirabilis	22	0	0	0	22
P. morganii	23	0	0	0	23
P. rettgeri	9	0	0	0	9
Providence (1)	11	0	0	0	11
(2)	47*	1†	0	2†	47
Other enterobacteria	75	65	42	10	0

* Toluene added to the buffer in these tests (see text).
† Weak reactions.

The decarboxylase tests showed that unlike most of the other enterobacteria tested, *Proteus* spp. and Providence cultures did not decarboxylate arginine or lysine (Table 2). To later tests toluene was added to the phthalate buffer as suggested by Møller (1954*a*) and under these conditions glutamic acid decarboxylase was detected in 45 of 47 Providence cultures, most of the *Proteus* cultures, and 7 of 8 *Escherichia coli* cultures (Table 3). Møller (1954*c*) described the decarboxylase reactions of a large number of enterobacteria which he has examined by a similar method. He increased the sensitivity of this test, and a simplified test with growing cultures (Møller, 1955), by using an acid medium to obtain the maximum amount of enzyme. We have found that some decarboxylase activity can be detected in *E. coli* cultures grown on Lemco agar at a neutral pH reaction and that the glutamic decarboxylase activity of *Proteus rettgeri* measured manometrically, is affected less than that of *E. coli* by the original pH reaction of the growth medium.

Table 3. *Glutamic acid decarboxylase shown by toluene-treated suspensions*

	No. tested	No. positive
P. vulgaris	5	4
P. mirabilis	9	8
P. morganii	7	1
P. rettgeri	6	6
Providence	47	45
E. coli	8	7

A washed suspension of *Clostridium welchii* used for the estimation of glutamic acid was tested by the micromethod and gave positive results for glutamic acid decarboxylase in 5–10 min. This mixture was then examined by paper chromatography, using 80 % phenol as solvent, and γ-aminobutyric acid could be detected. A suspension of *Proteus rettgeri* (NCTC 7477) gave a positive glutamic acid decarboxylase reaction in 30 min. and also a similar chromatogram.

DISCUSSION

Other workers have pointed out the relation between species of the genus *Proteus* and the Providence group (Kauffmann, 1951; Møller, 1953, 1954*a–c*; Ewing *et al.* 1954; Singer & Bar-Chay, 1954; Buttiaux, Osteux, Fresnoy & Moriamez, 1954). In general, our results are in agreement, but we have added more evidence in the tendency to swarm and in the production of H_2S by Providence strains, as well as the amino acid reactions. The addition of a known sulphur source to our medium accounts for the greater sensitivity of our test for H_2S (Clarke, 1953). Only a few Providence strains split urea, but as Singer & Bar-Chay (1954) showed, the urease-positive strains do not differ in any other respect from the urease-negative strains.

King (1953) described leucine and valine decarboxylases of *Proteus vulgaris* which were not present in other species of Enterobacteriaceae. We obtained positive results for almost all Proteus and Providence cultures by our microtest method. As this enzyme has a neutral pH optimum, and leucine is a good substrate for the deaminase, the leucine decarboxylation was probably obscured by its deamination. A washed suspension of *P. rettgeri* (NCTC 7477) was tested manometrically (pH 6·8, 0·025 M-phosphate buffer, gas phase N_2, with acid tip at the end of the reaction period). The suspension failed to decarboxylate leucine, but a small amount of CO_2 was produced from endogenous substrate by the cells alone. Under these conditions *P. morganii* (NCTC 3667) was shown to decarboxylate leucine, but *P. mirabilis* (NCTC 5887), which had given a doubtful reaction in Proom & Woiwod's amine test, also failed to decarboxylate leucine.

Proteus rettgeri was separated from other *Proteus* spp. by Proom & Woiwod (1951) because it gave negative results in a test for amines which were produced from valine and leucine in their medium, and did not require nicotinic acid for growth. Kauffmann (1953) went a step further and created separate genera for *P. rettgeri* (Rettgerella), *P. morganii* (Morganella) and Providence strains (Providencia), leaving Proteus with only two species, *P. vulgaris* and *P. mirabilis*. The similarities between *Proteus* spp. and the Providence group suggest a natural relationship, and we do not agree with Kauffmann (1953) that separate genera are required for these different species, an opinion which he revised in 1954. We think that our work adds to the evidence linking the Providence group to the genus *Proteus*. The first description of a Providence culture appears to be that of *Bacillus inconstans* by Ornstein (1921), which was amplified by Braun & Löwenstein (1923–4). Consequently the correct specific epithet will be inconstans, and the species should be designated as *Proteus inconstans* (Ornstein) nov.comb.

We are indebted to Dr W. H. Ewing for providing many of the Providence strains used, and for confirming the identity of *Bacillus inconstans*.

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The Capsule of the Diatom *Navicula pelliculosa*

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SUMMARY: The cells of *Navicula pelliculosa* become invested in a gelatinous capsule when they cease to divide. This may occur when the culture medium becomes depleted of some necessary element, such as silicon, phosphorus, or nitrogen. A method for the extraction of the capsular fraction has been devised, which is based on its solubility in 20% (w/v) NaOH. It is a polyuronide, apparently consisting solely of glucuronic acid residues.

According to Mangin (1908) Bailey in 1851 was the first to mention an organic membrane regularly associated with the siliceous valves of diatoms. Mangin showed, by the use of stains, that there was no cellulose present in the cell walls of diatoms, and stated that diatoms were distinct from all other organisms in their possession of a membrane composed of pectic compounds alone. He showed that, like pectin, the material stained with ruthenium red, methylene blue, safranin, neutral red, naphthyl blue, and alum haematoxylin. The 'pectic' membrane and external envelope remained after his diatoms were digested with hydrofluoric acid, which dissolved the siliceous skeletons. In addition to this membrane, gelatinous pads, stalks, tubes, or envelopes are characteristic of many diatom species. These external secretions have been considered to be of the same chemical nature as the pectic membrane, in that they give similar staining reactions (Liebisch, 1929). So far as the writer is aware, no chemical analysis of this pectic compound has been made hitherto.

Kützing's original description of *Navicula pelliculosa* (*Synedra minutissima β pelliculosa*) in 1849 made reference to a gelatinous capsule—'Individua in gelatina membranacea nidulantia'—which has given the organism its present specific name (Germain, 1935). Locker (1950) grew this species in culture and observed that the capsule stained with ruthenium red or safranin. She also referred to slime on the surface of ponds, which can be attributed to this organism when it is present in abundance.

Navicula pelliculosa (Bréb. ex Kütz.) Hilse has been independently isolated in pure culture. There is no evident capsule around actively dividing cells, but capsular material appears to accumulate after the cessation of cell multiplication. It can be made more visible by mounting the cells in nigrosin (see Pl. 1, fig. 1) or by direct staining with toluidine blue or methylene blue; in the latter cases some shrinking of the capsule occurs. The present paper deals with the extraction and partial characterization of this material, and with nutritional factors affecting capsule formation by the cells.

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Isolation of the organism

The strain of *Navicula pelliculosa* used in these studies was obtained by streaking a sample of water from a Connecticut pond on agar containing minerals but no organic supplement. Colonies were visible on the surface after a 4-day illumination period. Organisms from one of these colonies were suspended in sterile water and restreaked on a second plate, and by repetition of this procedure the diatom was isolated in pure culture. Tests for the presence of bacteria were carried out by inoculation into glucose Tryptone media and by microscopic examination. The diatom culture has been maintained on agar slopes in test tubes, with transfer to fresh media every 2 months.

Growth medium and culture methods

The mineral medium used contained the following concentrations of salts (%, w/v): K_2HPO_4, 0·02; $MgSO_4.7H_2O$, 0·02; $Ca(NO_3)_2.4H_2O$, 0·1 in distilled water. Traces of B, Mn, Zn, Cu, Mo and Fe were added. Silicon was added as potassium silicate ('Solution Silicate of Potash', Fisher, New York), giving a soluble silicon concentration of 1–3 mg. Si/l. (unless otherwise specified). At this concentration, silicon is limiting for growth but capsule accumulation is enhanced.

Mass cultures of organisms for chemical analysis were grown under aseptic conditions in 12 l. bottles containing 10 l. of inorganic culture medium. The organisms were illuminated by 'White' fluorescent tubes, and aerated with compressed air sterilized by passage through a cotton filter. Cultures for the study of the nutritional factors affecting capsule formation were grown in 125 ml. Pyrex Erlenmeyer flasks, containing 25 ml. liquid/flask. These flasks were shaken constantly at 60 oscillations/min. on a glass-bottomed extension attached to an Eberbach shaking machine. Illumination of 300 ft. candles was provided by four 'White' fluorescent lamps below the extension, and the position of the flasks was changed daily to ensure a uniform average illumination. All cultures were grown at 23°.

Preparation of organisms for extraction

The organisms from 10 l. of culture medium were allowed to settle, and the supernatant solution poured off. Sufficient HCl was added to the remaining suspension to dissolve precipitated calcium phosphates. The organisms were concentrated by centrifugation and the total wet weight of the sludge determined. From the wet weight and dry weight of a small sample, the dry weight of the whole mass of organisms was calculated. Yields of from 2 to 3 g. (dry weight) were obtained.

Extraction of the capsular material

Preliminary investigations revealed that the capsule material of *Navicula pelliculosa* was insoluble in cold or hot water. Attempts were made to dissolve the diatom capsule by following the standard methods employed for the extraction of angiosperm pectins (Loomis & Shull, 1937; Bonner, 1950). At each stage a sample of the insoluble cell residue was stained and examined microscopically; the extract was neutralized, when necessary, and treated with excess ethanol to precipitate any extracted capsular material.

The diatom capsule was insoluble in hot 0·5 % (w/v) oxalic acid (a solvent for protopectin), insoluble in 0·5 % (w/v) ammonium oxalate (a solvent for pectic acid), insoluble in Schweitzer's reagent, cupric-ammonium hydrate (a solvent for cellulose), slightly soluble in cold 5 % (w/v) NaOH, and rendered soluble by cold 20 % (w/v) NaOH. After dilution, neutralization, and filtration of the 20 % NaOH extract, and after addition of $CaCl_2$ (1 vol. M-$CaCl_2$ to 6 vol. extract) and ethanol (1 vol. of 95 % ethanol in water to 1 vol. extract), a gummy white flocculum precipitated. This precipitate was coagulated by heating, collected, washed with water and ethanol, dried and weighed. The dried material swelled rapidly in water without dissolving and was found to retain the staining properties exhibited by the capsule of intact organisms.

In subsequent analyses the organisms were first extracted with 80 % (v/v) ethanol in water to remove sugars, fatty constituents, some pigments, etc., then with 5 % (w/v) NaOH which saponified and removed most of the chlorophyll persisting after the ethanol extraction; finally the capsule was dissolved in 20 % NaOH. The yields obtained in several experiments showed that the capsule material accumulated as the culture aged, confirming the microscopic observations. The extracted capsular material represented 17 % of the dry weight of organisms from a 12-day culture; 37 % from a 20-day culture; 42 and 50 % from 27-day cultures.

Chemical characterization of the capsular material

Micro-Kjeldahl determinations for organic nitrogen, based on the method of Ma & Zuazaga (1942), were carried out on samples of extracted diatom capsule. Only 0·2 % nitrogen was found, corresponding to not more than 1·2 % protein and indicating that little or no mucoprotein was present.

Samples (10 mg.) of dried capsular material were hydrolysed at 100° for 5 hr. in sealed glass ampoules with 1 ml. 0·5 N-, 1·0 N-, 2·0 N-, or 4·0 N-H_2SO_4, or 87 % formic acid. The hydrolysates were diluted to 10 ml. and neutralized with $BaCO_3$ to about pH 5·0 (Congo red). The $BaSO_4$ and excess $BaCO_3$ were removed by centrifugation and the supernatant fluid evaporated to 1 ml. on a boiling water bath. Samples of these solutions were then chromatographed by descending migration. Drops of these solutions were applied with a wire loop 8 cm. from the edge of a sheet of Whatman no. 1 filter paper ($20\frac{1}{4} \times 18\frac{1}{2}$ in.). Standards of 4 mg. samples of sugars and uronic acids were run as controls. Migration was carried out in a 'Chromatocab', model A (Research Equipment Corporation, Oakland, California, U.S.A.) and the time of the run depended on the solvent.

The solvents used were: (1) *n*-butanol/ethanol/water, 4:1:5 (Flood, Hirst & Jones, 1948); (2) *n*-butanol/glacial acetic acid/water, 4:1:5 (Partridge, 1948); (3) acetone/water/formic acid, 9:1:1 (Evans & Mehl, 1951, modified by Dr C.

Partridge, unpublished). The developing reagents, used as spray, were: (1) 0·2 % ethanolic solution of naphthoresorcinol, acidified with 0·1 vol. of orthophosphoric acid (Bryson & Mitchell, 1951); (2) 3 % *p*-anisidine hydrochloride in *n*-butanol (Hough, Jones & Wadman, 1950).

In preliminary experiments with butanol/ethanol/water as solvent, the hydrolysate moved only a short distance relative to the control sugars, indicating the presence of a uronic acid. No pentoses or hexoses were detected. Samples of commercial galacturonic and glucuronic acids were run for comparison. Glucuronic acid lactone was partially converted to the free acid by hydrolysis with 0·5N-H_2SO_4 as described above. The capsule hydrolysate migrated at the same rate as did the known uronic acids, both in solvents (1) and (2). The R_F values obtained with these solvents are shown in Table 1. Capsular material hydrolysed with 2N-H_2SO_4 or 87 % formic acid also gave a spot which corresponded with the glucuronic lactone spot. The spot given by the hydrolysate of capsule material gave the same colour with *p*-anisidine as did the uronic acid controls (cherry red).

Table 1. *Relative migration distances (R_F values) of hydrolysis products of capsular material from* Navicula pelliculosa *and known markers in various solvent mixtures*

Compound	Solvent 1,* 21 hr.	Solvent 1, 21 hr.	Solvent 2, 18 hr.	Solvent 3, 11 hr., off end†	Solvent 3 5 hr.
Xylose	0·32	0·27	0·33	0·67	0·67
Galacturonic acid	0·22, forward trail	0·18	0·22	0·58	0·63
Glucuronic acid	0·04	—	0·20	0·63	0·62
Glucuronic lactone	0·41	0·35	0·42	—	0·76
Diatom capsule hydrolysed in:					
N-H_2SO_4	—	—	—	0·62	—
2N-H_2SO_4	0·08, 0·40	—	—	0·63	0·64, 0·73
4N-H_2SO_4	—	—	0·20	—	—
87 % formic acid	—	0·35, slight spot at 0·16	—	—	—

* See text (p. 164) for composition of solvent mixtures.
† R_F values estimated, based on migration of xylose.

To distinguish between glucuronic and galacturonic acids, solvent (3) was used. The results shown in Table 1 indicate that the capsule material does not consist of galacturonic acid, but might be glucuronic or mannuronic acid. Dr J. K. N. Jones (Queen's University, Kingston, Ontario, Canada) subsequently confirmed the presence of glucuronic acid residues only (personal communication).

Nutritional factors which affect capsule formation

Preliminary experiments indicated that capsule formation took place only at the end of the exponential phase of growth. To confirm this diatoms were grown in silicon-deficient, nitrogen-deficient, and phosphorus-deficient media

(Table 2); at intervals the density of growth was determined and the cells examined microscopically for capsule formation. From the results obtained (Fig. 1), it can be seen that in each case capsule formation occurred only when cell division was inhibited by depletion of the limiting element. Silicon determinations at the end of 15 days revealed 21·6 mg. Si/l. in the phosphorus-deficient culture, 7·3 mg. Si/l. in the nitrogen-deficient culture, and 0·2 mg. Si/l. in the control and silicon-deficient cultures.

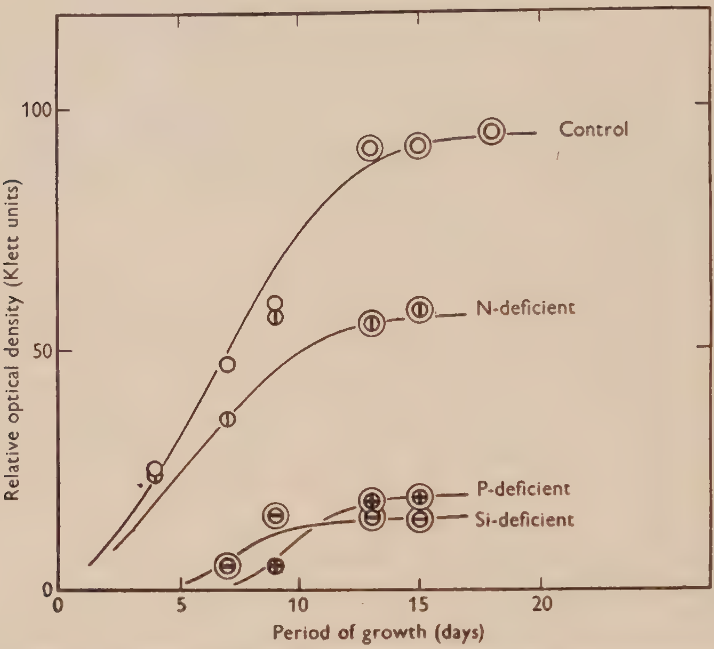

Fig. 1. Growth and capsule formation of *Navicula pelliculosa* in various nutrient media. Double circles indicate when capsules were visible microscopically. (The control flasks became Si-deficient after 10 days.)

When capsulated cells were transferred to a complete nutrient medium, cell division began again, and the cells so formed, lacking capsules, ultimately burst out and were liberated from the parental capsule.

Table 2. *Chemical composition of media employed in nutrition experiments with* Navicula pelliculosa

Compound	Control	Si-deficient	P-deficient	N-deficient
Potassium silicate	30 mg. Si/l.	3 mg. Si/l.	30 mg. Si/l.	30 mg. Si/l.
$MgSO_4.7H_2O$	0·2 g./l.	0·2 g./l.	0·2 g./l.	0·2 g./l.
$Ca(NO_3)_2.4H_2O$	1·0 g./l.	1·0 g./l.	1·0 g./l.	0·1 g./l.
K_2HPO_4	0·2 g./l.	0·2 g./l.	0·02 g./l.	0·2 g./l.
KCl	—	—	0·17 g./l.	—
$CaCl_2.2H_2O$	—	—	—	0·62 g./l.
Trace elements	1 ml./l.	1 ml./l.	1 ml./l.	1 ml./l.

DISCUSSION

The capsular polyuronide of *Navicula pelliculosa* can no longer be regarded as pectin, since it is a polymer of glucuronic and not of galacturonic acid. It remains to be determined in what way the uronic acid residues are linked in the natural polymer, and whether the latter is combined in some way with protein or with the silica walls, which might account for its peculiar insolubility in dilute alkalies.

It has been suggested that in various diatoms gelatinous secretions may play a role:

(*a*) In locomotion (see Fritsch, 1935). However, it is only the non-capsulated cells of *Navicula pelliculosa* which have been observed to exhibit motility.

(*b*) As a flotation device for planktonic forms (Naumann, 1925).

(*c*) As an attachment mechanism. In many diatom species the cells are enclosed in tubes, or adhere to one another or to the substrate by stalks or pads.

(*d*) As a protection against aquatic herbivores. Undigested and living cells of *Navicula* spp. were found in the excreta of water animals (Michaelis, 1915).

(*e*) As a mechanism for dissolving siliceous minerals such as kaolin (Vinogradov & Boichenko, 1942).

(*f*) As a means of protecting the cell against desiccation, due to the ability of the material to absorb many times its own weight of water. However, experiments by the present author indicated no such protection.

(*g*) As an extracellular food reserve. But encapsulated cells of *Navicula pelliculosa*, stored in the dark for a period of several weeks on a mineral medium, showed no decrease in the size of the capsules. The organisms apparently do not produce extracellular enzymes capable of hydrolysing their polyuronide capsules. Moreover, although these diatoms can grow and can form capsules in the dark on glucose, they are unable to grow when supplied with a hydrolysate of the extracted capsular material, or with glucuronic or galacturonic acid, as sole carbon source (Lewin, 1953).

(*h*) As an accumulation of a waste product. In certain bacteria (e.g. *Leuconostoc* spp.) polysaccharides are formed from carbohydrate units not utilized by the cell, and are secreted and accumulated in the form of a capsule.

The accumulation of capsular material by *Navicula pelliculosa* occurs when some nutrient in the culture medium becomes limiting, and growth is thereby inhibited. Organisms in such deficient media continue to photosynthesize, and part of the synthesized material appears ultimately as polyuronide, which gradually accumulates around the organisms. The presence of a capsule thus reflects a state of poor nutrient conditions; its function, if any, has not been determined. It is possible that the widespread occurrence of sheaths, capsules, mucilage tubes, etc., among various diatom species is likewise an indication of the limiting concentration of some essential nutrient in natural waters. These results are similar to those observed in *Aerobacter aerogenes* (Duguid & Wilkinson, 1953). In cultures of this bacterium, production of polysaccharide (intracellular, capsular, and slime fractions) increased when growth was limited by deficiency of an essential nutrient such as nitrogen or phosphorus, providing the carbon source was not depleted.

Diatoms are among the most abundant photosynthetic organisms in fresh water and the oceans, and the total annual crop of these algae may compare favourably with that of land plants (Allen, 1934; Steemann Nielsen, 1952). The polysaccharides associated with their cell walls may therefore be among the most abundantly formed organic materials in the world, and this may be of considerable significance in plankton ecology. It would therefore be of some importance to determine whether *Navicula pelliculosa* is exceptional in its production of a polyglucuronide capsule, or whether this material is of general occurrence in the membranes of the *Bacillariophyta*.

Some of the material presented in this paper was submitted in 1952 as part of a Ph.D. thesis at Yale University. The author wishes to express thanks to Dr Paul R. Burkholder and to Dr Ralph A. Lewin for many helpful suggestions, to Dr Ruth Patrick, Academy of Natural Sciences of Philadelphia, for the identification of the diatom species, to Dr Chester Partridge, Yale University, for aid and advice on techniques in paper chromatography, and to Dr E. G. Young for a critical reading of the manuscript. Dr J. K. N. Jones, Queen's University, Kingston, Ontario, Canada, examined a sample of the capsule material, and confirmed the presence of glucuronic acid in hydrolysates; his expert cooperation in this matter is gratefully acknowledged.

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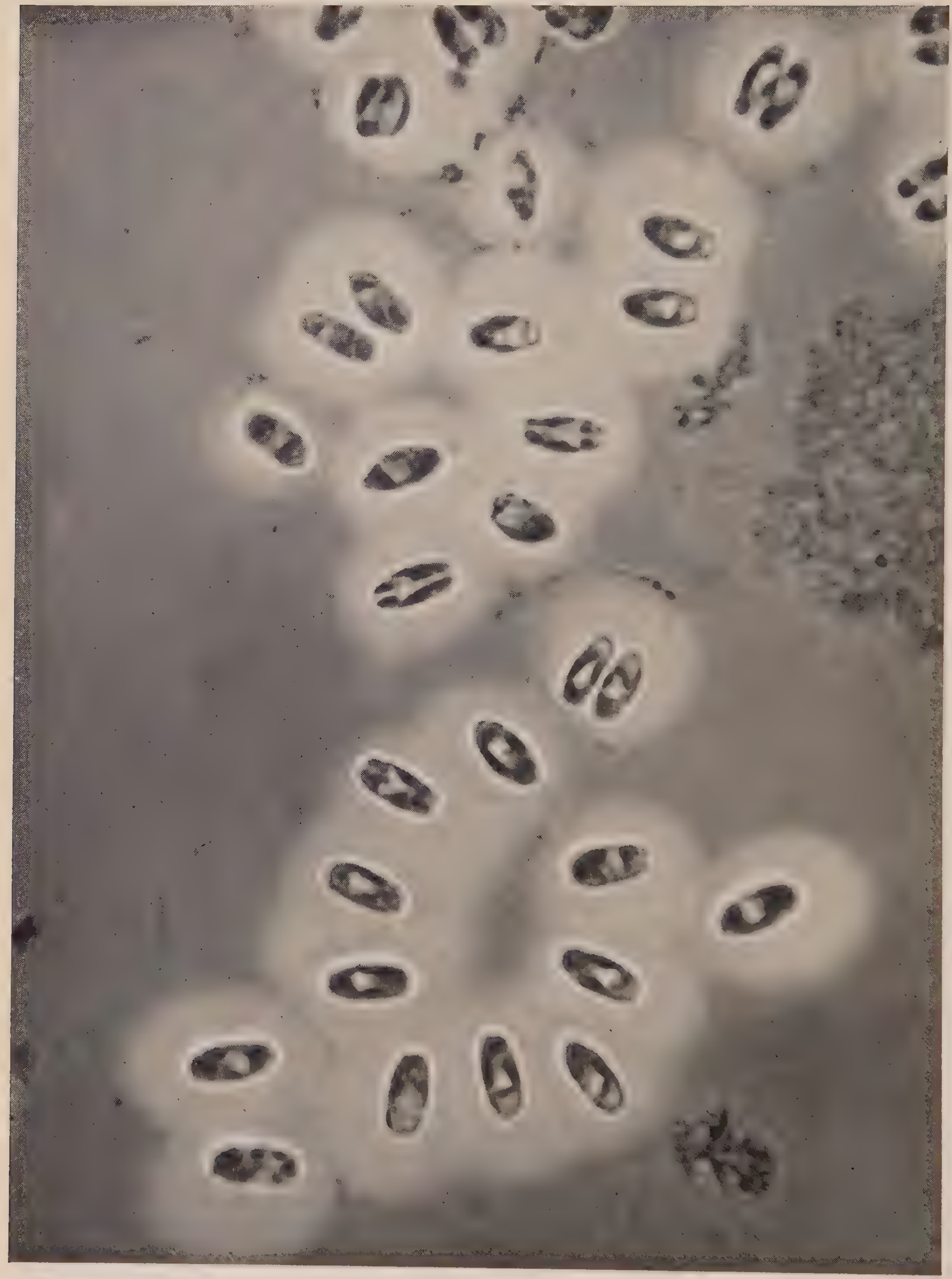

J. C. LEWIN—CAPSULE OF *NAVICULA PELLICULOSA*. PLATE 1

(*Facing p.* 169)

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EXPLANATION OF PLATE

Fig. 1. Photomicrograph showing capsules surrounding cells of *Navicula pelliculosa*. Nigrosin preparation. × 1750.

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Amine Production and Nutrition in the Providence Group

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SUMMARY: Fifty-six strains of non-lactose fermenting coliform organisms belonging to the Providence group were obtained from the National Collection of Type Cultures and examined for their nutritional requirements. Fifty-five of these strains formed a nutritionally homogeneous group. The simplest medium supporting growth was a basal salt medium with added glucose, glutamic acid and cystine. Pantothenate usually improved growth and appeared to be nearly essential for two strains. The remaining strain possessed the nutritional pattern of *Proteus morganii* and in this and other characters appeared to be a typical strain of that species. Twenty-two of the fifty-five strains gave a positive amine test using the ninhydrin test-paper technique; previously only *Proteus* species in the family Enterobacteriaceae had been observed to produce amines. The classification of the Providence group is discussed in the light of these findings.

Proom & Woiwod (1951) showed that the ability to produce amines by decarboxylation of certain amino acids was a constant and characteristic property of *Proteus* species. All of 47 strains of *Proteus vulgaris*, 75 strains of *P. mirabilis* and 10 strains of *P. morganii* were found to produce amines, whereas none of 257 strains representing other species of Enterobacteriaceae possessed this property. Although a wide selection of Enterobacteriaceae, including many strains of uncertain taxonomic status, were examined, the selection did not include any of the anaerogenic paracolon organisms classified by Kauffmann (1951) in the Providence group.

When *Proteus morganii*, strain 1721, of Stamp & Stone (1944) was examined, it was found to produce amines but did not decompose urea or require nicotinic acid as an essential metabolite. It was not therefore a typical *Proteus* species, and Kauffmann (1951) placed this strain in the Providence group. In consequence of this anomalous finding some Providence strains were obtained from the National Collection of Type Cultures and examined in particular for their ability to produce amines and for their nutritional requirements. The results are reported here and discussed in relation to classification.

MATERIALS AND METHODS

Strains. Fifty-six Providence strains were obtained from the National Collection of Type Cultures (NCTC 8993–9000 and NCTC 9190–9237). These strains were received by the National Collection from Dr W. H. Ewing.

Fermentation tests. Cultures were examined for their ability to ferment glucose, lactose, maltose, mannitol and sucrose. Fermentation tests were carried out in ¼ oz. screw-capped bottles containing *c.* 3 ml. of 1·0 % peptone water + 0·5 % of the relevant carbohydrate, with bromcresol purple as indicator. The cultures were examined at intervals up to 14 days.

Indole reaction was determined by Kovács's (1928) method using amyl alcohol as the solvent.

Voges–Proskauer reaction. Cultures were grown in glucose peptone water and the reaction determined by O'Meara's (1931) technique.

Methyl red reaction was determined in ¼ oz. screw-capped bottles containing *c.* 3 ml. glucose phosphate broth and the indicator added after 3 days incubation at 37° C.

Liquefaction of gelatin was determined in nutrient gelatin by incubation at 37° for 7 days, the tubes being cooled before reading the tests.

Decomposition of urea was determined in Christensen's (1946) medium and the results read after 4 and 24 hr.

H_2S *production* was determined by growth in 10 ml. nutrient broth contained in cotton-wool plugged test tubes with a strip of lead acetate paper as indicator; the results were read at intervals up to 6 days.

Phenylpyruvic acid reaction was determined by the method described by Henricksen (1950).

Amine production was determined by the ninhydrin test-paper technique described by Proom & Woiwod (1951).

Minimal nutritional requirements. The methods used were similar to those described by Knight & Proom (1950). The ammonia basal medium of Knight & Proom (1950) was used to supply ammonia, inorganic salts and trace metals. The medium was supplemented as required with 0·1 % glucose, L-glutamic acid (1·4 mg./ml. medium), L-cystine (0·02 mg./ml. medium), nicotinic acid (0·5 μg./ml. medium) and calcium pantothenate (1 μg./ml. medium). The glucose, amino acids and growth factors were added aseptically at ten times the required concentration after sterilization by Seitz filtration.

RESULTS

One strain, NCTC 9191 (Ewing, Tanner & Dennard's (1954) Providence 0 group 10), produced amines, required nicotinic and pantothenic acids as essential metabolites, actively decomposed urea, was indole-positive, VP-negative, MR-weakly positive, H_2S-positive, did not liquefy gelatin and produced acid with a small bubble of gas from glucose, but did not ferment lactose, maltose, mannitol or sucrose. This strain appeared to be a typical *Proteus morganii*. The results of the examination of the remaining 55 strains are summarized in Table 1.

For convenience the results are arranged in four groups, but it is not intended to imply that these represent four clearly defined subgroups. Indeed it is clear from Table 1 that there is little correlation between the variable characters. Some of the biochemical reactions, in particular indole production and gas production, were variable. A few strains were plated and individual colonies selected. With some strains both indole-positive and indole-negative variants occurred although repeated tests with the original cultures usually gave consistent results. It is possible that with a longer incubation period some strains reported here as indole-negative would have been found to give positive reactions. After plating, some colonies produced anaerogenic cultures and some produced a small bubble of gas with glucose. Repeated tests on the same cultures sometimes gave inconsistent results. Tests for H_2S production have recently been reviewed by Clarke (1953) and it seems clear that the conditions of the test can be arranged to produce almost any desired result. The method adopted here has been to test batches of nutrient broth with selected strains expected to give strongly positive, weakly positive and negative results. Only those batches giving the expected results were used. With this arbitrary method tests for H_2S production are often of taxonomic value. Using this technique *Proteus* species including *P. morganii* give strongly positive tests for H_2S within 24 hr. but the Providence strains and rettgeri are weakly positive and are usually negative at 24 hr., the difference between the two groups being clear-cut.

Table 1. *The nutritional and biochemical properties of 55 Providence strains*

Tests	Results			
Growth on basal salt medium supplemented with glucose, glutamic acid and cystine	+	+	+	+
Amine production by ninhydrin test-paper technique	+	+	−	−
Phenylpyruvic acid reaction	+	+	+	+
Fermentation reaction with				
Glucose	A or Asb	A or Asb	A or Asb	A or Asb
Lactose	−	−	−(1.Al)	−
Sucrose	Al	Al	Al	Al
Maltose	−	−(1.Al)	−(2.Al)	−
Mannitol	−	−	−(3.Al)	−
Indole production	+	−	+	−
Voges–Proskauer reaction	−	−	−	−
Methyl red test	+w	+w	+w	+w
Liquefaction of gelatin	−	−	−	−
Decomposition of urea	−	−	−(1+)	−
Number of strains	14	8	24	9

+ = positive reaction; − = negative reaction; A or Asb = production of acid with or without a small bubble of gas; L = late; W = weak. The results in brackets show the number of strains giving anomalous reactions.

All strains fermented glucose with or without a small bubble of gas. When gas was formed the amount was similar to that produced by *Proteus* species. Sucrose was fermented late and, with a few exceptions, lactose, maltose and mannitol were not fermented. All the strains were VP-negative, MR weakly positive, did not liquefy gelatin and gave a positive phenylpyruvic acid reaction. One strain was urease-positive.

The amine test was positive with 22 of the 55 strains. The results in all cases were clear-cut and reproducible. There was no correlation between amine production and the other variable characters within the group. The one urease-positive strain was amine-negative.

Nutritionally the strains formed a homogeneous group. All strains grew in the basal salt medium with glucose added as a possible source of energy and with added amino acids. The amino acid requirements were simple and satisfied by glutamic acid and cystine. Many cultures grew for two or three subcultures in the absence of cystine but required this amino acid for indefinite serial subculture. No vitamins were required as essential metabolites but pantothenate often improved growth and in two instances was nearly essential. The results with these two strains were rather equivocal, and it is probable that they could be maintained in serial subcultures in the absence of pantothenate. Nicotinic acid was not required; indeed, with many strains the addition of nicotinic acid to this simple medium inhibited growth.

DISCUSSION

Stuart, Wheeler, Rustigian & Zimmerman (1943), in their studies on the paracolon group of bacteria, described a serologically heterogeneous group of anaerogenic paracolons which they called type 29911. Stuart, Wheeler & McGann (1946) studied this group in more detail and described six biochemical subgroups which differed slightly in their fermentation reactions with carbohydrates. They included in this group certain other strains which had previously been described in the literature as *Shigella wakefield* (Berger, 1945) and the Sachs types B 81 and B 105 described as mannitol negative types of *Shigella* (Sachs, 1943) and it was suggested that type 29911 should be classified as a transitional group without specific or generic status. The resemblance to *Proteus* was in the fermentation and IMViC reactions, the ability to swarm on 1·0% agar plates after the strains had been conditioned by many serial subcultures in semi-solid agar, and in the possession of many minor and some major antigens found in the *Proteus* group. The fact that an occasional strain gave a weak, and an exceptional strain a strong, urease reaction was regarded as additional evidence of the relationship to *Proteus*.

Kauffmann (1951), in his collected studies on the Enterobacteriaceae, placed type 29911 and related organisms in a new group which he called the Providence group since many of the isolates studied by Stuart *et al.* (1946) were isolated from the vicinity of Providence (Rhode Island, U.S.A.). Kauffmann (1951) regarded this group as related to *Proteus* and stated that the only reliable biochemical difference between the Providence and *Proteus* groups was the failure of the former to decompose urea. Kauffmann (1951) classified rettgeri in the genus *Proteus* and stated that the only differences between rettgeri and other members of the genus were that rettgeri fermented mannitol and was sodium citrate-positive.

It has been shown by Henricksen & Closs (1938) and Henricksen (1950) that *Proteus* species can be differentiated from other enteric organisms by their ability to produce phenylpyruvic acid by oxidative deamination of L-phenylalanine. All *Proteus* strains were found to give a positive phenylpyruvic acid reaction and only an occasional strain of other Enterobacteriaceae gave a weak or exceptionally a strong reaction. Clarke & Shaw (1954) found that *Proteus*, rettgeri and Providence cultures gave positive phenylpyruvic acid tests; and only occasional weak reactions were found with other species in the family. A test for the development of alkalinity from leucine was described and found to be specific for *Proteus* spp. including rettgeri and Providence strains.

Further information is presented here which is of interest in the classification of *Proteus* and Providence species. Amine production occurred in a substantial minority of the Providence strains studied, whereas previously it had been found only with *Proteus* species. The spread-over of amine production into the Providence group does not detract from its value as a definitive generic character for the genus *Proteus*. In a sense it enhances its value since variable amine production occurs only in a group similar to *Proteus* in other respects.

The Providence group forms a nutritionally homogeneous group requiring for growth only inorganic salts, glutamic acid and cystine. It differs from the *Proteus* species in that it does not require nicotinic acid and from rettgeri in that this species will grow on inorganic salts and glucose (Proom & Woiwod, 1951). *Proteus*, Providence and rettgeri have three distinct basal nutritional patterns, and these patterns should be considered in relationship to their classification. The emphasis to be placed on nutrition in classification is partly a matter of opinion but more properly depends on the extent to which the patterns are characteristic and constant within the group studied.

The genus *Proteus* can be defined on the basis of a large number of characters. The characters differentiating this group from other Enterobacteriaceae were given by Proom & Woiwod (1951) as the ability to swarm on nutrient agar, the fermentation of glucose, but not lactose or mannitol, with production of a small volume of gas, decomposition of urea, marked H_2S production, amine production and a nutritional requirement for nicotinic acid. To these may be added a positive phenylpyruvic acid reaction. The loss of one or more of these characters would not necessarily exclude an organism from the genus and the possession of one or more of these characters would not justify its inclusion. The genus is defined on the pattern basis, that is on the assessment of as large a number of characters as possible, and then it represents a clearly defined group.

As soon as the generic definition is widened difficulties occur. The inclusion of rettgeri in the genus invalidates five of these characters, i.e. the production of small volumes of gas with glucose, failure to ferment mannitol, marked H_2S production, amine production and a nutritional requirement for nicotinic acid. The generic definition then rests on a somewhat ill-defined biochemical pattern, marked urease reaction, positive phenylpyruvic acid reaction and the development of an alkaline reaction with leucine. The last two tests were not considered when rettgeri was placed in the genus *Proteus* by Rustigian & Stuart (1943). If the Providence group is included then the generic definition becomes even more difficult. The biochemical pattern becomes more obscure; urease production is no longer an essential character and only a positive phenylpyruvic acid reaction and the development of an alkaline reaction with leucine remain. Positive phenylpyruvic acid reactions are known to occur in a minority of other strains in the family.

Because of a commendable reluctance to establish new genera, attempts are often made to 'press-gang' newly described species into existing genera and widen the generic definition. It is argued here that if the generic definition of *Proteus* is widened to include the Providence group and rettgeri then the definition becomes so vague that it is almost meaningless. It seems reasonably clear that the only alternative is to establish a new genus to include Providence and rettgeri.

My thanks are due to Mr E. Harris and Miss G. M. Trim for technical assistance.

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SINGH, B. & MITCHISON, D. A. (1955). *J. gen. Microbiol.* **13**, 176–184.

The Bactericidal Activities of Combinations of Streptomycin, Isoniazid, *p*-Aminosalicylic Acid (PAS), Oxytetracycline (Terramycin) and Viomycin against *Mycobacterium tuberculosis*

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SUMMARY: The bactericidal activities of mixtures of various chemotherapeutic drugs were tested against the H37Rv strain of *Mycobacterium tuberculosis* when growing in Tween albumin medium. Oxytetracycline (terramycin) antagonized the bactericidal activities of streptomycin, isoniazid and viomycin, whereas *p*-aminosalicylic acid (PAS) was slightly synergistic or did not influence their activities. Mixtures of viomycin and streptomycin were slightly less bactericidal than streptomycin alone, but more bactericidal than viomycin alone. Viomycin acted synergistically with isoniazid, and with isoniazid + streptomycin. The emergence of strains resistant to streptomycin, isoniazid or viomycin was not prevented in the cultures by the presence of oxytetracycline, probably because of its rapid inactivation. Drug resistance emerged in dual mixtures which included PAS, where the concentrations of streptomycin, isoniazid or PAS were low, but not when these concentrations were high. Resistant strains did not emerge in cultures containing viomycin + streptomycin or viomycin + isoniazid.

Streptomycin, isoniazid and *p*-aminosalicylic acid (PAS) are the compounds most commonly used chemotherapeutically in the treatment of tuberculosis and they were given simultaneously in pairs, or all three together, with the aim of preventing or delaying the emergence of resistant strains of tubercle bacilli. When the strains isolated from patients before treatment are resistant to two or more of these compounds, it has been suggested that combined treatment with compounds, which include oxytetracycline (terramycin) or viomycin, should be used. Hobby, Lenert, Donikian & Pikula (1951*b*) observed that oxytetracycline inhibited the growth of strains of *Mycobacterium tuberculosis*, both sensitive and resistant to streptomycin to an equal extent. They also found that it had limited activity in suppressing experimental tuberculosis in mice. Oxytetracycline alone has been found to have little therapeutic effect on patients with pulmonary tuberculosis (Pfefer, Hughes & Dye, 1952; Soderholm, 1952). However, Miller, Sands, Walker, Dye & Tempel (1952), Des Autels (1953), Miller, Sands, Gregory, Hightower, Weisser & Tempel (1954) and Rothstein & Johnson (1954) have shown that oxytetracycline + streptomycin was as effective as streptomycin + PAS or as streptomycin + isoniazid in preventing the emergence of streptomycin-resistant organisms. Furthermore, Stewart, Turnbull & Crofton (1955) showed that oxytetracycline + isoniazid effectively suppressed the emergence of isoniazid resistance in strains which were initially resistant to streptomycin, to PAS, or to both of these compounds.

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Viomycin was found to be equally bacteriostatic against streptomycin sensitive and resistant strains of tubercle bacilli by Steenken & Wolinsky (1951), Hobby, Lenert, Donikian & Pikula (1951*a*) and Karlson & Gainer (1951). Viomycin had an effect on experimental tuberculosis in guinea-pigs and mice comparable to that of a dose of streptomycin four times smaller (Steenken & Wolinsky, 1951; Youmans & Youmans, 1951). Drug resistance to viomycin was observed by Werner, Tompsett, Muschenheim & McDermott (1951). Clinical trials of viomycin + PAS (Hackney, King, Marshall, Harden & Payne, 1953; Pitts, O'Dell, Fitzpatrick, Dye, Hughes & Tempel, 1953; Hobby, 1953) indicated an action similar to that of streptomycin + PAS, but superior to that of streptomycin, PAS or viomycin alone.

Reasons for considering that the bactericidal activity of a chemotherapeutic substance or a mixture of such substances is one of the best tests of therapeutic efficiency have been given elsewhere (Mitchison, 1954; Singh, 1954). *In vitro* experiments on the bactericidal activity of mixtures of oxytetracycline + streptomycin or + penicillin against organisms other than tubercle bacilli showed that oxytetracycline inhibited the bactericidal action of the other component of the mixture (Jawetz, Gunnison & Speck, 1951; Gunnison, Coleman & Jawetz, 1950). Mackaness & Smith (1953) found that, *in vitro*, oxytetracycline abolished the bactericidal action of isoniazid against tubercle bacilli. The present communication reports an investigation of the bactericidal activity against *Mycobacterium tuberculosis* of mixtures of oxytetracycline + streptomycin, isoniazid or viomycin, and of viomycin + isoniazid, streptomycin or PAS. Observations have also been made on the ability of these mixtures to suppress the emergence of drug-resistant strains *in vitro*.

METHODS

The method of testing the bactericidal action of the compounds was described previously (Singh & Mitchison, 1954, 1955). The compounds were added to Tween albumin cultures of the H37Rv strain of *Mycobacterium tuberculosis* so that the final concentrations approximated to those found in the serum of patients treated with usual therapeutic doses. Surface viable counts on oleic acid + albumin agar were then carried out at intervals from these cultures. Sensitivity tests to streptomycin, isoniazid and PAS were done by the Medical Research Council (1953) method. Viomycin and oxytetracycline sensitivity tests were performed by methods similar to those used for streptomycin sensitivity tests.

RESULTS

Bactericidal activities

Fig. 1 shows the bactericidal activities of oxytetracycline 10 μg./ml., isoniazid 2 μg./ml. and of a mixture of these concentrations of oxytetracycline and isoniazid. Oxytetracycline alone had no bactericidal action, but inhibited the growth of the bacilli for 10 days. After 10 days growth occurred, presumably due to inactivation of the oxytetracycline (Bryson & Demerec, 1950; Regna & Solomon, 1950). Isoniazid alone had a well-marked bactericidal action, but in the presence of oxytetracycline there was no significant decrease in the viable count until after the 10th day.

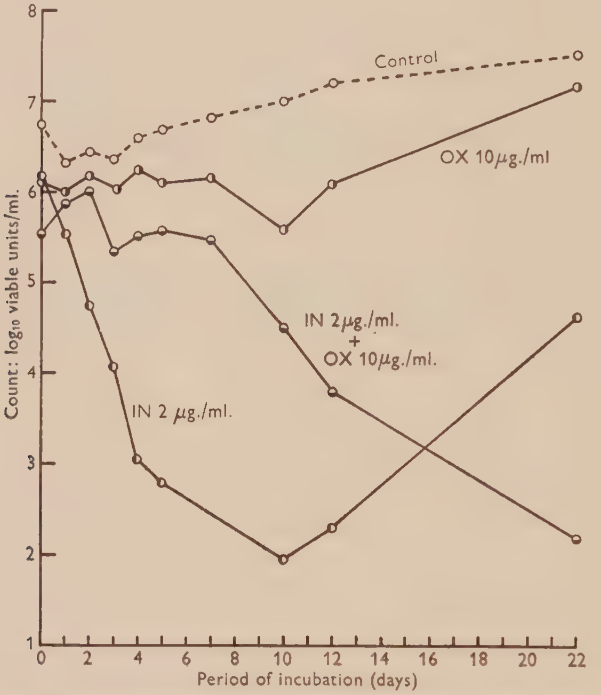

Fig. 1. **Bactericidal activities of isoniazid and oxytetracycline, alone and in combination. IN = isoniazid; OX = oxytetracycline.**

Fig. 2 shows an experiment with isoniazid 2 μg./ml. alone, or mixed with oxytetracycline 10 μg./ml., or PAS (sodium salt) 100 μg./ml. Similar mixtures with streptomycin 20 units/ml. were also set up. Here again, oxytetracycline prevented the bactericidal action of isoniazid for 7 days, but the bactericidal action of streptomycin was decreased to a lesser extent. In contrast, PAS had a slightly synergistic bactericidal action, more marked with streptomycin than with isoniazid.

Mixtures that included viomycin were set up in the experiment shown in Figs. 3–5. Fig. 3 shows the bactericidal activity of viomycin 40 μg./ml. alone and with oxytetracycline 10 μg./ml., or PAS (sodium salt) 100 μg./ml. For comparison, the culture containing isoniazid 2 μg./ml. is also included, and it can be seen that viomycin alone had approximately the same activity as isoniazid. Oxytetracycline again prevented the bactericidal activity of viomycin during the first 7 days, but PAS had no demonstrable antagonistic or synergistic effect.

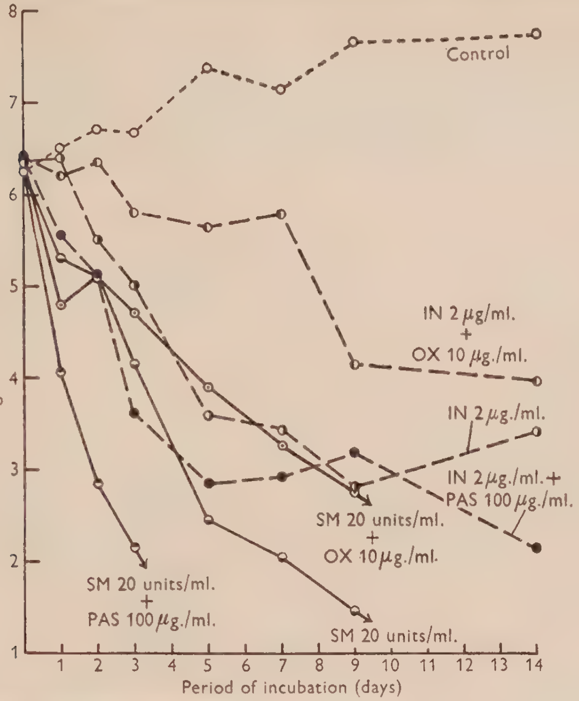

Fig. 2. Bactericidal activities of isoniazid or streptomycin, alone or in combination with oxytetracycline or PAS. IN = isoniazid; SM = streptomycin; OX = oxytetracycline; PAS = *p*-aminosalicylic acid (sodium salt).

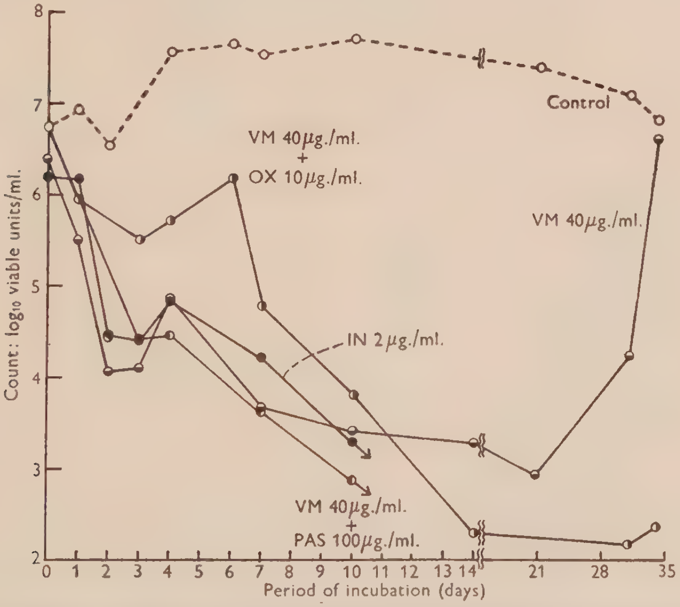

Fig. 3. Bactericidal activities of viomycin, alone or in combination with oxytetracycline or PAS. IN = isoniazid; VM = viomycin; OX = oxytetracycline; PAS = *p*-aminosalicylic acid (sodium salt).

Fig. 4 shows the activities of viomycin 40 μg./ml. alone, streptomycin 20 units/ml. alone and the two substances together. Viomycin was less bactericidal than streptomycin. In mixture, viomycin appeared to decrease the activity of streptomycin, although the degree of antagonism was so small as to be of doubtful significance; the mixture was, however, more bactericidal than viomycin alone.

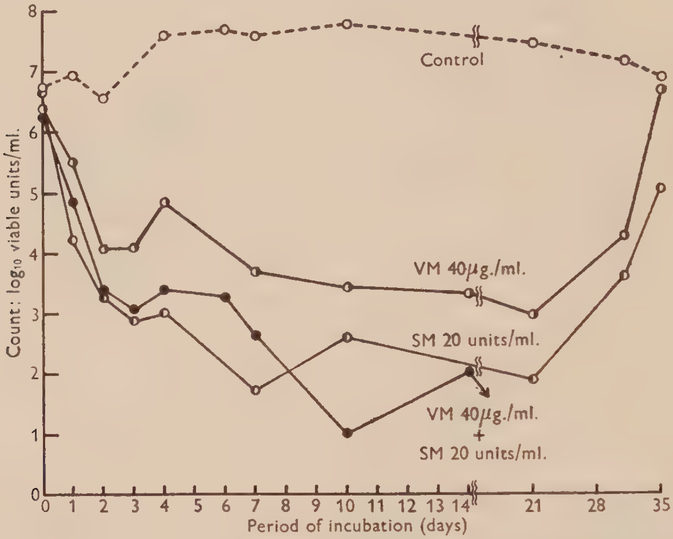

Fig. 4. Bactericidal activities of viomycin and streptomycin, alone and in combination. VM = viomycin; SM = streptomycin.

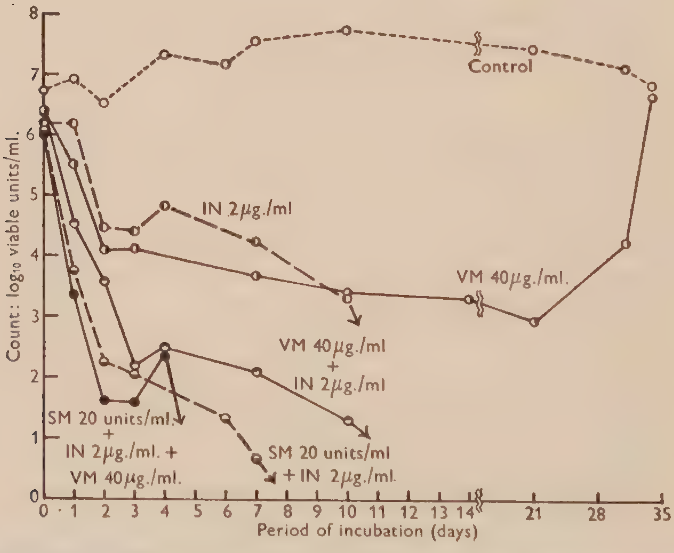

Fig. 5. Bactericidal activities of combinations of viomycin with isoniazid or isoniazid and streptomycin. IN = isoniazid; VM = viomycin; SM = streptomycin.

In contrast, viomycin + isoniazid was distinctly more bactericidal than viomycin or isoniazid alone (Fig. 5), and the mixture was only slightly less active than streptomycin + isoniazid. It can also be seen from Fig. 5 that the triple combination of streptomycin, isoniazid and viomycin was more actively bactericidal than any of the double combinations.

Resistance

As can be seen in Figs. 1–4, resistant organisms usually began to multiply in those cultures which contained streptomycin, isoniazid or viomycin, between the 10th and 21st days. The emergence of resistant organisms was investigated by preparing a series of cultures containing streptomycin or isoniazid alone or mixed with oxytetracycline or with PAS, in the same manner as for those cultures used in the bactericidal tests. These cultures were incubated and examined at intervals. The presence of macroscopic growth was noted and confirmed by subculture on to Lowenstein-Jensen slopes and on to nutrient agar slopes as a test of purity. Sensitivity tests were carried out on samples of the growths that eventually occurred. All cultures which had been in contact with streptomycin or isoniazid were found to show some degree of resistance to these compounds. On the other hand, no cultures showed resistance to oxytetracycline; the highest resistance ratio found was 2. The results obtained with these cultures are shown in Table 1. It can be seen that the addition of oxytetracycline 10 μg./ml. did not influence the emergence of organisms resistant to streptomycin or to isoniazid. On the other hand, the suppression of the emergence of resistant organisms by admixture with PAS was complete when PAS + streptomycin or PAS + isoniazid were present at high concentrations equal to the maximal concentrations found in the serum of treated patients. Lower concentrations of PAS + streptomycin or of

Table 1. *Appearance of macroscopic growth in cultures containing streptomycin, isoniazid, PAS or oxytetracycline*

Streptomycin (units/ml.)	Isoniazid (μg./ml.)	Sodium PAS dihydrate (μg./ml.)	Oxytetracycline (μg./ml.)	Period of incubation (days)	Total cultures	Cultures with macroscopic growth	
						Number	%
2	—	—	—	101–104	19	17	89
2	—	—	10	104	10	9	90
2	—	10	—	86	12	9	75
2	—	100	—	86	11	6	55
20	—	—	—	136	10	2	20
20	—	—	10	136	9	3	33
20	—	100	—	40	2	0	0
—	0·2	—	—	58	5	5	100
—	0·2	10	—	73	1	1	100
—	0·2	100	—	58	10	10	100
—	2	—	—	58–103	20	20	100
—	2	—	10	58	10	10	100
—	2	100	—	103	12	0	0

PAS + isoniazid were less effective in preventing the growth of resistant organisms; this confirms previous observations (Singh & Mitchison, 1955). Of the cultures shown in Fig. 3, those containing viomycin alone and viomycin + oxytetracycline both yielded strains resistant to viomycin (resistance ratios of 8 and 16) at 30 days. The culture containing viomycin + PAS yielded no growth at 30 days.

DISCUSSION

In confirmation of the observations of Mackaness & Smith (1953), oxytetracycline alone was found to have no bactericidal activity against the test organism *Mycobacterium tuberculosis*, strain H37Rv. Mixed with isoniazid or viomycin, oxytetracycline prevented their bactericidal action almost completely. These findings suggest that, in clinical practice, the eventual killing of the mycobacteria by these combinations would depend largely upon the host's defence mechanisms. Rist (1953) found that oxytetracycline + isoniazid was much less effective in the treatment of experimental infections in mice than was isoniazid alone. Tucker (1954) reported that viomycin + oxytetracycline was less effective in the treatment of human pulmonary tuberculosis than was viomycin + isoniazid, streptomycin or PAS. The *in vitro* antagonism between oxytetracycline and streptomycin was less marked. There is some evidence, although based on retrospective clinical comparisons, that streptomycin + oxytetracycline is of the same order of effectiveness as streptomycin + PAS in the treatment of pulmonary tuberculosis in man (Miller *et al.* 1952, 1954).

The eventual growth of resistant mycobacteria in cultures containing streptomycin + oxytetracycline, isoniazid + oxytetracycline or viomycin + oxytetracycline was presumably due to inactivation of the oxytetracycline. Thus it is not possible to conclude that in clinical practice such mixtures would be ineffective in delaying or preventing the emergence of resistance, since active concentrations of oxytetracycline would be maintained in the lesions by repeated dosage. Nevertheless, the closely similar incidence of resistance to streptomycin which developed in cultures exposed to streptomycin alone or to streptomycin + oxytetracycline suggests that the slight bactericidal activity of the mixture affected only those organisms which were sensitive to streptomycin and not the few streptomycin-resistant mutant organisms which were present initially in the cultures. Thus, during treatment with streptomycin + oxytetracycline, the streptomycin-resistant organisms initially present would be prevented from growing by the oxytetracycline, but might remain as a potential menace to the host.

In contrast to the antagonistic activity of oxytetracycline and in confirmation of earlier work (Singh & Mitchison, 1955), PAS, which also has no bactericidal activity under the conditions employed, was found to be either slightly synergistic with each of the three compounds, streptomycin, isoniazid and viomycin, or not to influence their bactericidal activity. Streptomycin and isoniazid have been shown to have little bactericidal activity on resting cultures (Middlebrook & Yegian, 1946; Tison, 1950; Schaeffer, 1954). It is possible that oxytetracycline may also produce resting conditions in cultures, so accounting for its antagonistic activity. On the other hand, Sievers (1949) and Rist (1953) claim that tubercle bacilli exposed to PAS multiply for a few generations before bacteriostasis occurs; the bactericidal compounds may be able to kill the organisms during this phase of multiplication.

Hobby *et al.* (1951 *a*) reported that subinhibitory concentrations of viomycin were sometimes sufficient to decrease significantly the concentrations of streptomycin required to inhibit growth of tubercle bacilli. No synergistic bactericidal activity was, however, found in the present work in the mixture viomycin 40 μg./ml. + streptomycin 20 units/ml., nor was any synergistic effect observed when the two compounds together were used in lower concentrations in a further unreported experiment. The mixture of these two compounds, however, prevented the development of resistant strains. A mixture of viomycin + PAS also prevented the growth of viomycin-resistant strains, which may explain the observation of Pitts *et al.* (1953) that, in the chemotherapy of pulmonary tuberculosis, viomycin + PAS was superior to viomycin or streptomycin alone. Viomycin + isoniazid was more bactericidal than viomycin or isoniazid alone. The degree of synergism was, however, less than that observed with streptomycin + isoniazid. The mixture viomycin + streptomycin + isoniazid was more bactericidal than any two together.

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The Use of Ammonium Sulphate in the Production of Lysates of Staphylococcus Phage K of High Titre

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SUMMARY: In the production of high titre staphylococcus phage K lysates from concentrated liquid bacterial cultures the upper limit of phage concentration appears to be at about 10^{10} particles/ml. This concentration, the 'ceiling titre', can be raised to 4×10^{11} particles/ml. by the addition of ammonium sulphate to the medium after phage infection. It is concluded that this effect depends upon the ability of ammonium sulphate to lower the rate of adsorption of phage upon host cells, thus preventing a high rate of phage loss by adsorption and inactivation towards the end of lysis. The possibility is discussed that this mechanism may explain some puzzling observations in lysing systems.

In the course of experiments with staphylococcus phage K, large amounts of bacteriophage were required for purification (Hotchin, 1954); for this, attempts were made to improve the phage yield above that usually obtained in broth lysates. It was noticed that in a rich medium aeration was the limiting factor for high bacterial growth rate. However, when improved methods of aeration were used, although the bacterial growth (measured turbidimetrically) was considerably increased, corresponding increases in the phage titre were not obtained. Even when conditions of phage inoculation (e.g. growth phase of host cells, infective ratio) were varied widely, there appeared to be an upper limit ('ceiling titre') of about 10^{10} plaque-forming units/ml., above which the phage concentration did not rise, in spite of complete lysis of very dense bacterial cultures. It seemed probable that under these conditions at the ceiling titre, phage was being lost at a rate approximating that at which it was being formed. Adsorption to an inactivating agent (possibly bacterial cells) was considered to be the most likely reason for phage loss, and experiments were conducted to attempt to prevent this loss and to test the hypothesis.

METHODS

Organisms and medium. The Oxford strain of *Staphylococcus aureus*, NCTC 6571, and staphylococcus bacteriophage K were used throughout. Hartley tryptic digest broth was used as nutrient medium. Ammonium sulphate was 'Analar' grade.

Bacteriophage assay. This was made by plaque counts on the surface of nutrient agar (1 %) plates.

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RESULTS

Specific antibacterial serum was tested in an attempt to prevent adsorption of progeny phage upon other infected cells of the culture which had not yet lysed. The agglutination titre of the serum was 1/1280 for host cells, and it was used at final concentrations of 1/640, 1/64 and 1/6. Ammonium sulphate was known to slow down or prevent the adsorption of T 2 coliphage on to *Escherichia coli* (French, Graham, Lesley & Rooyen, 1952). Consequently, it was hoped that it might similarly affect the staphylococcal system. Therefore ammonium sulphate at a final concentration of 5 % (w/v) was tested for its ability to increase the titre of a staphylococcal phage K lysate when added after infection. The serum or ammonium sulphate solution was added to the culture 5 min. after phage infection of 10^9 host cells/ml. by phage K at $1{\cdot}5 \times 10^9$ particles/ml. The results of such an experiment are shown in Table 1.

Table 1. *The effect upon final phage titre and appearance of the lysate of certain additions to cultures of* Staphylococcus aureus *with and without phage*

Concentration of *Staphylococcus aureus* 10^9 organisms/ml. Infective ratio (phage/host cells) 1·5:1.

Tube no.	Presence of phage (+ or −)	Additions to system (final concentration)	Time of addition of additive relative to phage infection (min. + or −)	Phage titre at 24 hr. $(+10^{10})$	Appearance of lysate
1	−	Nil	.	.	Very turbid
2	−	Serum dil. 1/64	.	.	Clear, organisms agglutinated and settled. Approx. same turbidity as no. 1 on shaking
3	−	$(NH_4)_2SO_4$, 5 % (w/v)	.	.	As no. 1
4	+	Nil	.	1·7, 2·0*	Clear, slight sediment
5	+	Serum dil. 1/640	+5	3·3	Clear, slight sediment
6	+	Serum dil. 1/64	+5	2·2	Clear, slight sediment
7	+	Serum dil. 1/6	+5	2·3	Clear, slight sediment
8	+	$(NH_4)_2SO_4$, 5 % (w/v)	+5	6·7	Clear, coarse sediment
9	+	Serum dil. 1/64	−2	3·3	Clear, slight sediment
10	+	$(NH_4)_2SO_4$, 5 % (w/v)	−2	<0·1	Very turbid (no lysis)

* duplicate tubes

The results obtained in several experiments showed that suitable additions of ammonium sulphate did increase the titre of the final lysate, while antiserum did not significantly affect the titre. Turbidity time curves showed that the ammonium sulphate concentration used had very little inhibitory effect upon the growth rate of the host, and addition of ammonium sulphate before adding the phage regularly prevented phage multiplication and lysis. These results are in keeping with the notion that the ammonium sulphate acts by slowing adsorption. This was tested by measuring the velocity constant K

(Adams, 1950) of the rate of adsorption of phage upon host at 37° in broth containing varying amounts of added ammonium sulphate. Chloroform-killed staphylococci were used at a final concentration of 10^8/ml. in the experimental tubes. Tubes received 5 ml. broth, 1 ml. suspension of cocci and different volumes of 5 % neutral ammonium sulphate in saline. The volume in each tube was made up to 10 ml. with saline, and 0·1 ml. phage suspension containing 10^9 plaque-forming units/ml. added. The mixtures were then allowed to stand for 1 hr., and the unadsorbed phage then assayed. The results are shown in Fig. 1 which is typical of several experiments. The curve shows that increasing concentrations of ammonium sulphate markedly decreased the adsorption rate of the phage upon its host. The optimal effect upon phage yield occurred beyond the higher limit of the experimental range shown, where the velocity constant was less than 1/100th of its normal value. Further experiments indicated that the optimal final concentration of ammonium sulphate for maximum lysate titre was at a concentration of *c.* 0·1–0·2 M (1·5–2·5 %, w/v) according to the batch of broth used. Addition of ammonium sulphate at this concentration 2 min. after phage inoculation regularly gave lysates with titres of between 1 and 4×10^{11} particles/ml. Small variations in the ammonium sulphate concentration sharply affected the rate of lysis and the final titre. Lysis in the presence of ammonium sulphate was slower than in control cultures without it. One-step growth curves showed a slight increase

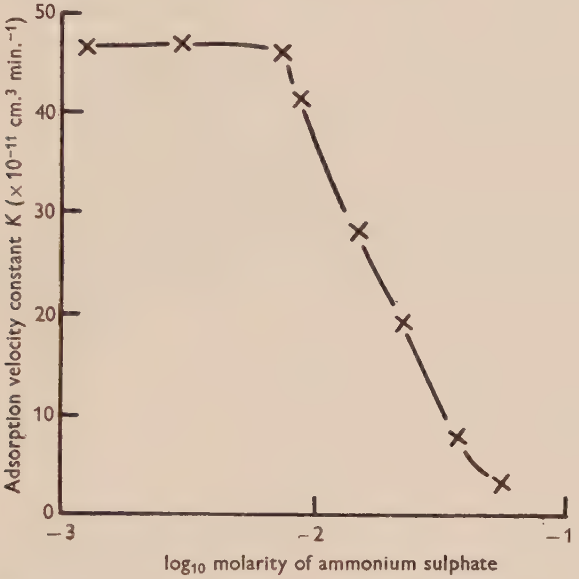

Fig. 1. The effect of varying concentrations of ammonium sulphate upon the velocity constant (K) of the adsorption of staphylococcal phage K on to host staphylococci. $K = 2{\cdot}3/(B)t \times \log P_0/P$ where (B) = concentration of bacteria (no. cells/ml.), t = time (min.), P_0 = phage particles/ml. at zero time, P = phage particles/ml. not adsorbed at time t min.

in the latent period when the optimal concentration of ammonium sulphate was added, the burst size being the same as in controls without added ammonium sulphate. The increase in latent period was not considered sufficient to account for the slower lysis of cultures.

DISCUSSION

The results described here are in keeping with the hypothesis proposed; ammonium sulphate can decrease the rate of adsorption of staphylococcus K phage upon its host *Staphylococcus aureus*, and in suitable conditions this effect is paralleled by a marked increase in phage yield, after a delayed lysis. It is considered that the first effect is the cause of the second and that the following mechanism holds. The concentration of free titratable phage in a system of multiplying host cells is the resultant of two opposing processes: the liberation of phage into the medium or phage production (P) and the loss of plaque-forming units or phage loss (L). The phage loss L initially occurs almost entirely by adsorption upon uninfected host cells, and later by adsorption upon uninfected, infected and dead host cells and bacterial debris, plus any other causes of phage inactivation or death. Immediately after a culture is infected with phage $L > P$ (since P at this time is zero) thus accounting for the usual fall in free phage titre at this stage. As phage multiplication proceeds P increases rapidly, becoming greater than L, and the concentration of free phage rises. In concentrated systems it seems that the increase of P becomes limited, possibly by nutritional factors, before the lysis of the culture. Phage loss by adsorption is proportional to the concentration of free phage, so that L increases until $L = P$. This appears to occur in the system studied at phage concentrations of *c*. 10^{10}/ml., this being the ceiling titre at which phage production is balanced by phage loss. By adding ammonium sulphate to phage the adsorption velocity and consequently phage loss is decreased. Under these conditions the same rate of phage production will only be balanced by phage loss when there is a much higher concentration of free phage to compensate for the decrease in adsorption velocity; i.e. the ceiling titre will be higher.

The inability of host cell antiserum to increase the titre by decreasing phage adsorption is surprising in view of its effect in preventing phage adsorption to host in the T coliphages (Delbrück, 1945). This difference was not investigated further, although it suggests that the phage adsorption site on the host cell is not covered by host-cell antiserum in the staphylococcal system.

A similar mechanism to the one proposed appears to be operating in experiments described by Bradley & Boyd (1952). The results of these workers indicate that phage released by the lysing fraction of a culture of lysogenic *Salmonella typhimurium* is lost to the extent of 99·9 % by re-adsorption on to phage-immune cells; this re-adsorption was prevented by Cetrimide at 1/10,000 dilution.

When lysis of the staphylococci occurred after the ceiling titre had been reached, there was no further rise in phage K titre; this phenomenon offers an alternative explanation for the results of Farrant & Rountree (1953) with staphylococcus phage 3A. They concluded that release of this phage from its host preceded and was not synchronous with optical clearing of the culture, the release being due to bursting of the cells, while the cell membranes left were considered able to contribute to the same extent as the intact cells to the turbidity of the culture. However, simple rupture of staphylococcal membranes which allows cell contents to escape is known to result in a tenfold or greater decrease in turbidity (Hotchin, 1954). Similarly, phase-contrast microscopy readily differentiates ruptured from unruptured staphylococci (Hotchin, Dawson & Elford, 1952). These differences are presumably due to the high refractive index of the bacterial cytoplasm when in the concentrated intracellular state. It is hard to visualize a change in permeability of the staphylococcal membrane which would allow the release of relatively large phage particles while retaining the bacterial cytoplasm. An alternative explanation for their results is that in Farrant & Rountree's system the ceiling titre was reached before the culture cleared optically.

The observations described are obviously important to consider when large yields of phage are desired. It is possible that the effect may occur in animal virus systems, especially when conditions of growth (e.g. suspended cell cultures) approximate those of fluid bacteriophage cultures.

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Nutritional Requirements and Biochemical Activities of Brewery Lactobacilli

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SUMMARY: Four strains of lactobacilli, isolated from the contaminant flora of English top fermentation brewers' yeasts, were found to be heterofermentative. Two strains were tentatively identified as *Lactobacillus brevis*. For growth, all the strains required exogenous supplies of a number of growth factors of the vitamin B complex, a pyrimidine, at least one purine and at least 14 amino acids. The ability of hypoxanthine to substitute for both adenine and guinane in one strain of *L. brevis* is especially notable. L-Arginine, essential for the growth of all strains, increased the rate and final amount of growth in glucose medium of both strains of *L. brevis*, when it was supplied in relatively high concentration. Suspensions of the organisms converted arginine into ornithine, ammonia and a small amount of citrulline. Maltose, fructose and L-arabinose were better substrates for the growth of all strains than glucose, which, as major source of energy, supported little or no growth of the two unidentified strains. All the strains transformed fructose into mannitol. The rate and extent of growth of most strains on hexoses and the rate of growth of all strains on maltose were increased by the presence of acetate. Whereas the strains of *L. brevis* formed only little acid from glucose, all strains produced considerable amounts of volatile and non-volatile acids from L-arabinose.

Certain lactic acid bacteria have long been recognized as beer spoilage organisms. However, unlike their counterparts which inhabit dairy products there have been few studies of the nutrition of these bacteria. Recently, Russell, Bhandari & Walker (1954) surveyed the vitamin requirements of 34 strains, comprising 10 species of brewery lactic acid bacteria. The present work is restricted to four strains isolated from pitching yeasts of top fermentation breweries, and reports on their nutritional requirements with respect to growth factors of the vitamin B complex, purines, pyrimidines, amino acids and major sources of energy, and some biochemical activities.

METHODS

Organisms. The strains used in this work were isolated from the contaminant flora of samples of English top fermentation yeasts obtained from three different breweries. Stock cultures were carried on liquid unhopped malt wort. The strains were designated L3, L4, L5 and L6.

Media. (*a*) Unhopped malt wort (sp.gr. 1·036) was prepared from brewers' pale ale malt. (*b*) The complete medium for strains L3 and L4 was as described by Chamberlain & Rainbow (1954) for *Lactobacillus brevis*, with the addition of 1 mg. each of adenine and guanine/100 ml.; for convenience this will be denoted as CR medium. This CR medium was also used for strains L5 and L6, but the glucose was omitted and 1·8 g. L-arabinose added. Except where otherwise stated, media and test additions were sterilized in admixture by heating in the autoclave momentarily to 15 lb./sq.in. pressure.

Inocula. (*a*) For tube tests, transfers were made from stock wort cultures into 6 ml. of appropriate complete semi-defined medium. After incubation at 28° for 16–24 hr., the cells were harvested by centrifugation, washed three times with portions each of 6 ml. of sterile 0·85 % saline and resuspended in a further 6 ml. of sterile saline. A portion of this suspension was diluted 150 times with sterile saline and after shaking well, one drop of this dilute suspension was used to inoculate each tube.

(*b*) For work with suspensions of organisms, inocula were prepared by transferring from stock cultures to 6 ml. portions of malt wort medium, incubating at 32° and washing the crop three times as above. The washed organisms were then suspended in the appropriate solutions for metabolic study.

(*c*) Inocula for larger cultures (60 ml.) in malt wort were grown as in (*b*) and for those in CR media as in (*a*). Each 60 ml. culture was inoculated with 3 ml. of the resultant subculture, without washing (for malt wort cultures) or after washing three times and resuspending as in (*a*) (for cultures in CR medium).

Tube tests (6 ml. cultures) for growth response were carried out as described by Chamberlain & Rainbow (1954).

Experiments with suspensions of organisms were carried out at 28°, the substances to be metabolized being added as sterile aqueous solutions at pH 6·5, to organisms which had been harvested and washed as described above. After incubation, the organisms were centrifuged and the supernatant fluids examined by paper chromatography or by other procedures as described.

Determination of fermentation acids. After incubation for 120 hr., the total volatile acids formed in 60 ml. cultures were determined as described by Fred, Peterson & Davenport (1919). Total acids were determined either by direct titrations of measured samples of the fermented liquid or, when fermentation was carried out in the presence of calcium carbonate, by determination as calcium oxalate of the calcium in solution. The figures for non-volatile acid were obtained by difference.

Paper chromatography. The appropriate supernatant fluids were examined by single run descending papergrams. The upper layer of a mixture of *n*-butanol + ethanol + water (4:1:5, v/v) was used to separate fructose from mannitol and the components were revealed by spraying with $AgNO_3$ reagent (Hough, 1950). For runs involving arginine, the conditions and solvents were those used by Partridge (1948), amino acids being detected by spraying with 0·1 % ninhydrin in *n*-butanol saturated with water.

RESULTS

Characteristics of the strains

Morphological. Each isolate consisted of non-motile, non-sporing, Gram-positive rods.

Cultural. The strains grew copiously in unhopped malt wort, producing a silky turbidity which settled after extended incubation to a powdery deposit. Good growth also occurred in stab cultures in malt agar.

Temperature and pH. Tube tests showed that all strains grew well in malt wort at pH 5·8 and 32–35° and in appropriate semi-defined medium at pH 6·2 and 26–28°.

Biochemical. All the isolates were catalase-negative, VP-negative and produced lactic acid when grown on malt wort. Lactic acid was isolated by the method of Franzen & Stern (1921). Polarimetric examination of the ethereal extracts of the free acid and the behaviour of the water of crystallization on heating the crystalline zinc salt showed that DL-lactic acid was formed in each case. Other biochemical activities of the strains are reported below.

Identification. Strains L3 and L4 are tentatively identified as strains of *Lactobacillus brevis*, and strains L5 and L6 may also belong to the same species, differing from L3 and L4 especially with regard to capacity to utilize glucose for growth (see below).

Nutritional requirements

Growth factors, pyrimidines and purines. The requirements of the strains for individual growth factors of the vitamin B complex and for pyrimidines were determined by growth tests in which each test substance was omitted in turn from an otherwise complete CR medium. To determine the purine requirements, growth tests were carried out in purine-free CR medium, the purines (adenine, hypoxanthine, guanine and xanthine) being added singly and in all possible combinations.

Table 1. *Growth factor requirements of brewery lactobacilli*

Growth tests were made at 28° in CR medium containing 1 % each of glucose and L-arabinose. Observations were qualitatively the same over the incubation period of 48–148 hr. The result of omission of a growth factor is signified as follows: E (essential) = little or no growth; S (stimulatory) = growth restricted to 50 % or less of maximum growth; O (not essential) = little or no restriction of growth.

Growth factor	L3	L4	L5	L6
Thiamine	S	S	S	S
Pyridoxin	O	O	O	O
Pantothenate	E	E	E	E
Nicotinate	E	E	E	E
Riboflavin	E	O	O	O
Biotin	S	S	S	S
Pteroylglutamic acid	E	E	E	E
Uracil	E	E	E	E
Purines	E	E	E	E

Table 1 summarizes the results. All strains showed absolute requirements for pantothenate, nicotinate, pteroylglutamate (PGA), a purine and uracil. In addition, the growth of all strains was severely restricted by omission of thiamine or biotin. For strains L3, L5 and L6, cytosine or orotic acid, but not thymine, could replace uracil. For strain L4, uracil was replaceable, only after a period of induction, by cytosine, but not by orotic acid or thymine. The purine requirement of strains L3, L5 and L6 was met by either adenine, hypoxanthine, guanine or xanthine. However, strain L4 was unusual in that its requirement for two purines (adenine and guanine or xanthine) could be met by hypoxanthine alone. This fact, which was applied in another connexion by Chamberlain & Rainbow (1954), suggests that hypoxanthine is a common precursor of both adenine and guanine in strain L4. The general pattern of growth factor requirements was similar for all strains, but strain L3 was unique in requiring added riboflavin, to which it responded sharply over the range 0–1·5 mμg./ml.

Note on casein hydrolysate. The casein hydrolysate in the CR medium used for the above tests was manifestly free from pantothenate, nicotinate, PGA, riboflavin, uracil and purines. Tests with other suitable micro-organisms showed that the casein hydrolysate was also substantially free from thiamine, biotin, pyridoxin and *p*-aminobenzoate, although it is recognized that the preparation may still contain quantities of these factors (in particular, of biotin) sufficient to permit partial or full growth of strains L3, L4, L5 and L6.

Amino acids. To determine the requirements of each strain for individual amino acids, growth tests were carried out for 4–5 days at 28° in CR medium, the casein hydrolysate of which was replaced by a mixture of 18 amino acids (Henderson & Snell, 1948) each of which could be omitted in turn from the otherwise complete medium. The amino acids used in these tests were DL-isomers except as stated below.

The results may be summarized as follows:

(*a*) Essential in all cases: L-arginine, L-aspartate, L-glutamate, isoleucine, L-leucine, phenylalanine, L-proline, serine, threonine, tryptophan and valine.

(*b*) Alanine, glycine, L-histidine, L-lysine, methionine and L-tyrosine were either essential or markedly stimulated the growth of all strains, except as recorded in (*c*) below.

(*c*) Not essential: for strain L3, alanine and L-histidine; for strain L4, L-lysine and L-histidine.

Utilization of carbohydrates for growth. The capacity of a number of sugars, sugar alcohols and acetate to support growth was determined by tube tests in which the test substance was added individually to CR medium devoid of carbohydrate and acetate. The following results (Table 2) are noteworthy:

(*a*) Mannose, D-arabinose, sucrose, lactose, raffinose and acetate did not promote the growth of any strain and sorbitol and mannitol were at best only feebly utilized.

(*b*) Glucose was not utilized for growth by strains L5 and L6 as a sole source of carbon and/or energy, and although strains L3 and L4 grew on it, growth (especially during the first 48 hr.) was inferior to that on maltose, fructose or L-arabinose, all three of which were utilized by all strains.

(*c*) Galactose was utilized for growth, albeit poorly, by all strains.

These tests were repeated in the presence of 32 mg. $CH_3COONa.3H_2O$/ml. The following effects of acetate were observed. Growth of strains L3 and L4 on glucose and on galactose, and of strain L4 on fructose, was enhanced in the early stages by the presence of acetate. The final amount of growth of all strains on L-arabinose or on glucose + L-arabinose was lower in the presence of acetate. During the first 72 hr., the growth of all strains on maltose was strongly stimulated by acetate, but the final (168 hr.) amount of growth was little affected. Indeed, the turbidity of cultures of L3, L4 and L5 on maltose and acetate rose to a maximum in about 3 days, falling appreciably thereafter.

Table 2. *Utilization of carbohydrates for growth by brewery lactobacilli*

Growth tests were made at 28° in basal CR medium free from carbohydrate and acetate. Test substances were added as follows (mg./ml.): hexoses and sugar alcohols, 10; pentoses, 8·3; disaccharides, 9·5; raffinose, 9·7; $CH_3COONa.3H_2O$ (adjusted to pH 6·2), 32. The Spekker reading (168 hr.) of control tubes containing no test substance was 0·05 or less.

Relative degrees of growth (Spekker readings)	Strain of lactobacillus			
	L3	L4	L5	L6
	Additions to basal CR medium			
>1·20	Glucose + L-arabinose	Glucose + L-arabinose	L-Arabinose	—
0·80–1·20	Fructose or maltose	Fructose, maltose, L-arabinose, xylose or ribose	Ribose*, fructose or maltose	Fructose or glucose + L-arabinose
0·40–0·80	L-Arabinose, glucose, galactose, xylose or ribose*	Galactose* or glucose*	Glucose + L-arabinose, xylose or galactose	L-Arabinose, maltose, xylose* or ribose*
0·20–0·40	Sorbitol		Mannitol or sorbitol	Galactose or sorbitol
<0·20	Mannitol	Sorbitol or mannitol	Glucose	Glucose or mannitol

Not utilized by any strain: mannose, D-arabinose, sucrose, lactose, raffinose, acetate

* Signifies growth only after a lag period of 3 or more days.

The rate of growth of all strains on pentoses appeared to depend on some chemical change, induced by thermal sterilization, during which a stimulatory substance or substances was produced by interaction between pentose and a constituent or constituents of the medium. Thus, the growth of all strains was more rapid when sterilization was carried out with pentose in admixture with the other medium constituents than when the pentose and the other constituents were sterilized separately and then mixed when cold.

Biochemical activities

Types of fermentation. The molecular ratios of non-volatile to volatile acid formed by each strain after growth in malt wort varied from 3·3 to 5·9 or from 4·1 to 4·8 when $CaCO_3$ was present (Table 3). These values were low compared with those expected from homofermentative lactobacilli and indicated that our strains were heterofermentative. Strain L3 was selected for further examination, by similar means, for the type of fermentation effected in acetate-free CR media containing either glucose or L-arabinose. From glucose, only small amounts of acid were formed, the ratio of non-volatile to volatile acid being approximately 2:1. Much larger amounts of total acids, and especially of volatile acid, were produced from L-arabinose, the corresponding ratio being nearly 1:1 (Table 3; cf. Gest & Lampen, 1952).

Transformation of fructose. Cultures of strains L3, L4, L5 and L6 in CR medium containing 1·8 % fructose as sole carbohydrate, were grown at 28° for 144 hr. The presence of mannitol in the culture fluids of all strains was demonstrated by paper chromatography. This was confirmed by growing strain L3 on 60 ml. CR medium (containing 1·8 % fructose instead of glucose) for 144 hr. The method of Peterson & Fred (1920) was used to isolate from the culture filtrate 125 mg. of a substance which resembled an authentic specimen of mannitol in melting point (found, 166°; authentic specimen, 167–168°; mixed melting point, 166–167°) and in chromatographic mobility.

Table 3. *Acid formation by strain L3*

60 ml. cultures were grown for 120 hr. at 32° (in malt wort) or at 28° in CR medium, devoid of acetate and containing either 5 % glucose or 4·5 % L-arabinose. The ratio (*a*)/(*b*) assumes that the acids concerned are monobasic.

	Acid formed			
	Total	Non-volatile (*a*)	Volatile (*b*)	Ratio (*a*)/(*b*)
	ml. 0·10 N-acid/10 ml. culture			
Malt wort	5·76	4·93	0·83	5·9
Malt wort + $CaCO_3$	18·22	14·81	3·41	4·3
Glucose medium	1·25	0·83	0·42	2·0
Arabinose medium	11·35	5·96	5·39	1·1

Transformation of arginine. The rate and final amount (after 168 hr.) of growth of strains L3 and L4 in CR medium containing glucose as sole carbohydrate was considerably increased by the addition of 1–2 mg. L-arginine/ml., but not by DL-citrulline and/or DL-ornithine. The stimulatory action of arginine was not apparent when L-arabinose was also present in the medium. When washed cell suspensions of strains L3, L4, L5 and L6 were allowed to metabolize solutions containing 4–8 mg. L-arginine/ml. and the resultant supernatants were examined by paper chromatography, ninhydrin-positive substances identical in mobility with ornithine and citrulline were shown to be formed by all strains. The presence of citrulline, which was found only in small amounts, was confirmed by use of the *p*-dimethylaminobenzaldehyde spray of Dent (1948). The transformation of arginine by suspensions of organisms proceeded in the absence of glucose and growth factors and was accompanied by a change of pH from 6·2 to 8·6; ammonia was detected by Nessler's reagent in the supernatant or in the distillate from the supernatant made alkaline with KOH.

Dent's spray also serves to detect urea. Although 20 μg. urea was readily detected on the chromatograms by this method, the spot occupying a position between those of arginine and ornithine, no urea was detected in a portion of metabolized supernatant corresponding to 40 μg. L-arginine. Urea was also not detected by the xanthydrol test applied directly to the supernatants. No significant decomposition of urea was detected when washed organisms were incubated at 28° for 20 hr. with urea solution (1 mg./ml.).

That the substance formed from arginine was indeed ornithine and not lysine (which possesses the same chromatographic mobility under the conditions used) was confirmed by chromatography using the same *n*-butanol/ethanol/water solvent, but carried out under the acid conditions created by the presence of 1:1 glacial acetic acid + water mixture in the cabinet. Under these conditions, the substance derived from arginine was distinct from lysine, and identical with authentic ornithine. By similar methods, suspensions of strain L3 were shown to convert citrulline partially to ornithine, whilst ornithine was not attacked.

DISCUSSION

The lactobacilli which inhabit diary products are recognized as nutritionally exacting organisms, particularly with respect to their requirements for exogenous amino acids, purines, pyrimidines and growth factors of the vitamin B complex. The present work shows that some actual or potential beer spoilage lactobacilli are equally fastidious in these respects. Whilst the general pattern of requirements for growth factors shown by our strains agrees with that found by Russell *et al.* (1954), our strains differ in their absolute requirement for pteroylglutamic acid which was not required by any of the strains of Russell *et al.*

Our strains are heterofermentative lactobacilli, forming little acid from glucose but considerable amounts of acid from L-arabinose. They show certain unusual preferences with respect to utilization of carbohydrate (presumably required as a source of energy). Thus, only two strains utilized glucose for growth, whilst all strains grew well on L-arabinose, fructose or maltose. It thus appears that growth on maltose does not involve preliminary hydrolysis of the maltose molecule to glucose. The availability of maltose may reflect a nice adaptation of these lactobacilli to their natural environment (beer) the predominant carbohydrate of which is maltose. It is also of possible evolutionary interest that all the strains are capable of utilizing galactose for growth, if only to a limited extent.

Apart from the function of L-arginine as an essential amino acid, the stimulation of growth in glucose medium of strains L3 and L4 by relatively high concentrations of L-arginine suggests that the cleavage of the arginine molecule provides these organisms with a source of energy or some important intermediate, such as metabolic CO_2, which may also be derived from L-arabinose since the stimulatory effect of arginine is not apparent when L-arabinose is present.

The transformation of L-arginine to ornithine and ammonia by all strains is of interest in view of the reports of the arginine dihydrolase activities of other lactic acid bacteria (Hills, 1940; Oginsky & Gehrig, 1952) and because the ability to effect this transformation has been suggested as a physiological test in the classification of lactobacilli (Briggs, 1953). Although inconclusive, our evidence favours the hypothesis that our lactobacilli transform arginine by the dihydrolase system. The alternative (Krebs) mechanism requires the formation of urea as an end product by cells which do not possess urease activity.

The relatively feeble metabolism of citrulline to ornithine by suspensions of strain L3 accords with the observations of Oginsky & Gehrig (1952) who showed that, although acetone-dried cells of *Streptococcus faecalis* formed ammonia and CO_2 as rapidly from citrulline as from arginine, intact organisms effected only a slow breakdown of citrulline as compared with arginine.

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Haptene Substances in Culture Media for Lactobacilli

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SUMMARY: Tomato juice Yeastrel Tween 80 broth was found to contain two haptene substances, one a component of tomato juice and the other of Yeastrel. These caused difficulties in the use of lactobacillus antisera. Methods were devised for eliminating the effects of these haptenes.

Non-antigenic constituents of bacteriological media have been found on occasion to stimulate the production of antibodies when organisms grown in the media were inoculated into animals, presumably owing to adsorption of the substances onto the bacterial cells (e.g. Sordelli & Mayer, 1931*a*, *b*; Morgan, 1936; Glynn & Holborow, 1952). To produce antisera for work on the serology of lactobacilli (Sharpe, 1955) strains of lactobacilli for inoculation into rabbits were grown in the tomato juice Yeastrel Tween 80 broth (TDB) described by Briggs (1953). The antisera obtained from rabbits after several courses of inoculation with lactobacilli were found to react by precipitin test with HCl extracts of all the species of lactobacilli tested, completely obscuring the specific reactions. Sterile TDB medium also reacted with these non-specific antisera and tests on the different components of this medium showed that only the tomato juice gave a positive reaction. The non-specificity was thus due to a constituent of tomato juice which was adsorbed on to lactobacilli grown in the medium; then when these were used as antigen, antibodies for tomato juice were produced. Such sera reacted with the HCl extracts, as organisms for extraction were also grown in TDB medium. When rabbits were inoculated over a period of several months with tomato juice or TDB medium alone no antibodies for tomato juice were detected in the sera, indicating that the material in the tomato juice was a haptene and not a complete antigen. When lactobacilli for inoculation into rabbits were grown in TDB medium without Tween 80, the production of antibodies for tomato juice was delayed but eventually they appeared. Growth of some strains of lactobacilli was very poor in this medium. Absorption of tomato juice antisera with organisms of any heterologous strain of lactobacilli grown in TDB medium removed the tomato juice antibodies from the serum but also sometimes removed antibodies specific for lactobacilli (Sharpe, 1955).

Dialysis of tomato juice against distilled water showed that the haptene did not dialyse through a cellophan membrane. However, a medium of similar composition to TDB medium but prepared with the outer solution which had passed through the membrane gave a sparser growth, equivalent to the growth in TDB medium without tomato juice. Growth-promoting substances had evidently also been retained by the membrane, or so diluted as to be ineffective. Attempts to free lactobacilli grown in TDB medium from the tomato haptene before preparing HCl extracts were unsuccessful, neither could it be separated from these extracts by ethanol. As the removal of tomato juice antibodies from the sera or of the tomato juice antigen from HCl extracts of organisms proved impractical, the difficulty was overcome by growing lactobacilli for rabbit inoculation in a modified TDB medium, namely, without tomato juice, and using the complete TDB medium for growing organisms to be extracted for antigen for *in vitro* tests. The tomato juice antigen in the extracts was thus rendered ineffective, since the sera did not contain haptene antibodies. With this method, however, non-specific antibodies again appeared in the sera, although the reaction was less potent than previously, and it took longer for such antibodies to appear. This effect was traced to the Yeastrel, used in compounding the TDB medium, which gave rise to Yeastrel antibodies. This reaction was eliminated by omitting Yeastrel from the TDB medium used to grow the organisms to be used for preparing extracts.

Subsequently therefore as a routine, the effects of these haptenes were eliminated as follows: lactobacilli for rabbit inoculation were grown in a medium containing Yeastrel but no tomato juice and lactobacilli for preparing antigen extracts in a medium containing tomato juice but no Yeastrel. Since tomato juice and yeast extracts are increasingly used for culture media similar difficulties might arise in serological work with other organisms and might be avoided in the same way.

Lactobacilli grown in TDB medium appear readily to adsorb haptenes from the medium, and the fact that the presence of tomato juice antibodies in sera was very much delayed by the absence of Tween 80 from the medium suggests that this substance may have had some effect on the adsorption. In this connexion it is of interest that Middlebrook & Dubos (1947) found that tubercle bacilli grown in a medium containing Tween 80 adsorbed the Tween and inoculation of these organisms produced anti-Tween sera which reacted with Tween 80 by precipitin test and agglutinated tubercle bacilli grown in the medium. Contrary to these findings anti-Tween sera were not demonstrated with the lactobacilli used here: no antisera precipitated with Tween 80 or agglutinated organisms grown in the Tween containing TDB medium.

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CORRIGENDUM

In Moore, W. B. and Rainbow, C. (1955) *J. gen. Microbiol.* **13**, 190–197.

On p. 196, lines 3 and 4:

for: 'was confirmed by chromatography using the same *n*-butanol/ethanol/water solvent, but carried out under acid conditions'

read: 'was confirmed by chromatography using phenol + water solvent, carried out under acid conditions'

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Natural Selection in the Microbial World

The Second Marjory Stephenson Memorial Lecture

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It will be unnecessary, I trust, to dwell upon my gratitude to the Society for inviting me to deliver the second lecture in memory of Marjory Stephenson. I realize that my good friends in England are responsible for initiating and supporting the move that led to the final invitation, and that I owe this honour largely to their efforts.

The choice of an appropriate subject has been very difficult. This was mainly the result of having talked myself out during an extended lecture tour in the United States last year. After much contemplation and consultation I decided on a discussion of natural selection in the microbial world, partly because the topic holds a great deal of fascination and promise, partly also because it represents the other extreme end of the spectrum of research activities in microbiology that Marjory Stephenson had outlined in 1945 (Woods, 1953). It deals with the behaviour of micro-organisms in mixed cultures under natural conditions, and with the factors operative in the struggle for survival.

The general microbiologist recognizes the existence of a vast variety of micro-organisms, differing from one another in morphological respects, in developmental history, or in physiological and biochemical characteristics. To the scientific mind this very fact poses two fundamental questions. One is concerned with the manner in which this diversity came about; the other with the problem how the numerous types can maintain themselves in nature. I believe that at present there is no valid reason for thinking about this situation otherwise than in terms of the concepts developed, about a century ago, by Charles Darwin to account for the occurrence of the multitude of higher plants and animals. The main feature of the Darwinian approach is the introduction of the principle of natural selection. It implies that the environmental conditions determine which types can best compete with others, and thus survive. Granting the presence in a population of individuals with different characteristics, Darwin thus suggested a mechanism for the persistence of the 'fittest'.

That this mechanism also operates in the microbial world has been amply illustrated by many pertinent investigations which have, furthermore, provided information concerning the nature of specific factors involved in the selection process. It is the intent of the present paper to review some aspects of these studies. I shall not discuss the manner in which differences may originate among the individuals of an initially homogeneous microbial population. This is all the more unnecessary because an important contribution to this phase is supplied by the symposium on 'Adaptation in Micro-organisms', organized by your Society a few years ago. Suffice it to say that two fundamentally different processes have been found to occur, in protozoa, algae, fungi, yeasts and bacteria, resulting in the appearance of variants. They are: (1) adaptations, in the sense of changes of properties in response to environmental influences, without affecting the genetic composition of the organisms; (2) mutations and hybridizations, whereby the genotype is modified. It seems reasonable to assume that the latter processes have been largely responsible for the appearance of progressively more aberrant types, and in this manner have supplied the material upon which natural selection could act.

Many types of micro-organism appear to have persisted in nature for a comparatively long time. This follows from the fact that they can be found to-day, and recognized as identical with microbes encountered and described decades or even centuries ago. We may thus conclude that there must exist ecological niches which permit such organisms to maintain themselves in competition with others. In addition, the particular ecology of a specific microbe must differ from that of all others, and the requisite conditions must, from time to time, be realized in nature. Otherwise the organism would eventually disappear completely. It is one of the pious hopes of the medical profession that this situation might be accomplished with respect to some of the pathogenic micro-organisms, although there is also evidence to the effect that natural selection may counteract efforts in this direction.

Ecological studies aim at supplying the data on which to base an interpretation of the manner in which environmental factors determine the outcome of the struggle for existence. Progress in this direction has been very slow for the higher plants and animals; in most cases it has not been possible to reach conclusions extending beyond those of a very general nature. One may, for instance, explain the barrenness of certain areas as a result of the lack of sufficient moisture to enable plants to grow. And since animals are, directly or indirectly, dependent on plants as their ultimate source of food, the absence of animal life can thus also be accounted for. But investigations of a more refined nature have only recently been initiated, especially in the completely air-conditioned greenhouses at the California Institute of Technology, under the direction of F. W. Went. The results so far obtained indicate how much more may be expected from an extension of this work. To mention a few illustrative examples: the peculiar property of the seeds of certain desert plants to germinate only after a second rainfall of a definite magnitude is currently attributable to the presence in the seeds of substances which inhibit germination. Not until these have been removed by adequate leaching can the seeds sprout. This behaviour ensures that at the time of germination the water-level in the soil will be sufficiently high to permit further development to the point of fruition. Seeds that germinate after the first moistening cannot develop to this stage, and plants that behave in this manner are doomed to extinction in the desert environment.

A similar situation has been discovered in connexion with temperature and light. Some higher plants can complete their life cycles only if during the night the temperature regularly falls below a definite maximum; others if the duration of the periods during which they are exposed to light and darkness bears a definite relationship. The inability of such plants to flower and fruit under different circumstances tends to restrict their natural distribution to regions where the requisite conditions are met. Even though the physiological and biochemical events that govern flower- and fruit-formation may not be understood as yet, the results mentioned indicate the direction in which future research should move to reach a better comprehension of the regulatory mechanisms, and hence of the ecological relationships.

In studies on animal ecology, too, an occasional glimpse of salient environmental factors has been gained. An illustration is provided by the studies of Dobzhansky on the correlation between special environmental factors and the distribution of geographically distinct races of fruit flies. In collaboration with Mrak and El Tabi it has been established that different races of Drosophila display marked differences in their preference for specific yeasts, and that the occurrence of the latter is determined by the types of plants found in the environment. Thus a hitherto unavailable clue has been discovered which is apt to lead to a better understanding of Drosophila ecology.

Unfortunately, the relationships between the characteristics of an environment and the flora and fauna found therein must often be deduced from observations made at a time when the organisms are already present in large numbers. This is not always a satisfactory guide to an interpretation of ecological factors, because at such a time the environment may have been considerably modified by the activities of the organisms themselves. A simple case may illustrate this.

In many instances the growth of phytoplankton in a body of water is limited by the supply of minerals, particularly nitrogen compounds and phosphate. Since these substances are removed at an accelerated rate as the number of organisms increases, it stands to reason that at the time of maximum plankton growth the mineral content may be at a minimum. It would obviously be a fallacy to conclude from the chemical composition of the water at this time that absence of minerals is the cause of plankton development. In view of present knowledge, it is unlikely that anyone would confuse the cause-and-effect relationship in this case. But the situation is not always so simple.

It follows that a more dependable approach calls for observations prior to, as well as during, the appearance of specific organisms, so that an accurate picture of the sequence of events can be obtained. In this manner ecological relationships may be inferred with less ambiguity, and these can be subsequently tested by special experiments. Instructive in this connexion are the studies of Barker on the succession of phytoplanktonic components in marine bays (Barker, 1935). Fertilization of the sea water with minerals through land drainage or upwelling frequently causes the appearance of an abundant diatom flora, which is superseded by the development of dinoflagellates in equal profusion. The regularity of the sequence—relative paucity of phytoplankton, fertilization, appearance of diatoms, and, finally, of dinoflagellates—suggested that the growth of the diatoms so modifies the environment that it becomes more suitable for the competitive growth of dinoflagellates. One important factor determining the succession is the decrease in the concentration of nutrients during the diatom bloom. Experiments with pure cultures have revealed that diatoms grow more rapidly than dinoflagellates as long as the concentration of nitrogen compounds and phosphates exceeds a certain value. Below this level the growth rate of dinoflagellates can be the greater; in fact, these organisms develop with undiminished vigour at concentrations some hundred times lower than those needed for an optimal rate of diatom multiplication.

Although simple ecological observations may sometimes be difficult to interpret unambiguously, a satisfactory choice between alternatives may often be reached by a judicious extension. A striking case in point is furnished by the ecology of the sulphur bacteria. These organisms are encountered in environments containing hydrogen sulphide, and many of them can be easily recognized because they contain conspicuous inclusion bodies in the form of sulphur droplets. For many years the ecological relationship had been interpreted to mean that the sulphur bacteria are the cause of the hydrogen sulphide production. This view was supported by the copious liberation of hydrogen sulphide when masses of sulphur bacteria are stored in the laboratory. Nevertheless, an opposite inference was theoretically possible, and Winogradsky, aware of this possibility, carried out his masterly experiments which eventually led to the revolutionary concept of chemo-autotrophy (Winogradsky, 1887, 1888*a*). By showing that the growth of the sulphur bacteria is dependent on the presence of hydrogen sulphide, he finally resolved the ambiguity of the ecological situation. And, though it must be admitted that the novel interpretation was proved by straightforward physiological experiments, it should here be emphasized that ecological studies played a significant role. For as soon as the alternative explanation had been conceived, Winogradsky realized that it implied a verifiable consequence; if sulphur bacteria grow in a special environment because it contains hydrogen sulphide, the organisms should also be found in places where this constituent is present as a result of non-microbiological activities, such as in sulphur springs. The inference was tested and found to be correct; sulphur bacteria are found in all sulphur springs.

But once the ecological situation had thus been clarified, a new problem had to be faced. The fact is that there are many different types of sulphur bacteria, and this, in turn, implies that these types must require special conditions, different for each one, for their continued existence. Hence it becomes necessary to define these conditions in order to provide the basis for a better understanding of the mechanism of natural selection in this group of micro-organisms.

A beginning has been made in this respect. The sulphur bacteria can be divided into two distinct groups, comprising the coloured and the colourless representatives, respectively. The former are anaerobes and depend on a supply of radiant energy as well as of hydrogen sulphide. They can therefore develop only in places where light is available and where the sulphide concentration is maintained at a sufficiently high level to preserve anaerobic conditions. Moreover, in this group a further ecological segregation occurs under the influence of differences in sulphide concentration. In crude cultures the green sulphur bacteria consistently develop closer to the region of hydrogen sulphide production than do the red and purple sulphur bacteria, which indicates greater tolerance for the toxic substrate on the part of the first-mentioned organisms. Differences in tolerance for hydrogen sulphide exist even among the representative types of the red and purple sulphur bacteria, and it is thus reasonable to invoke concentration gradients as an important factor governing the distribution, and hence the natural selection, of the individual types. It may here be mentioned that these conclusions have been substantiated by the outcome of experiments of a sort to be discussed presently, viz. with elective cultures (van Niel, 1931; Larsen, 1952, 1953).

Much remains to be done, however, before we shall be in a position to specify accurately the conditions under which each one of the many readily distinguishable types can maintain itself in nature. At present we are, for example, completely ignorant of the factors that permit the occasionally observed mass development of the red sulphur spirilla. A more complete knowledge of their ecology should go far towards solving the problem of culturing these interesting organisms; as far as I know, not a single representative of this group has yet been isolated in pure culture, nor has it been possible even to propagate them in the laboratory in crude cultures.

Still more difficult is the situation concerning the colourless sulphur bacteria. Despite the fact that the conspicuous filamentous forms, Beggiatoa and Thiothrix, are often encountered in nature, we know little about the environmental factors that govern their occurrence, except that they are found in places where hydrogen sulphide and oxygen are present. The tantalizing Thiovulum, too, has long been refractory to more detailed studies, although Dr Wijler's recent experiments bring a glimmer of hope that the picture will be different in the not too distant future (unpublished; personal communication).

Ecological considerations long ago led Winogradsky to the conclusion that the brown, ferric hydroxide-encrusted bacteria of the Leptothrix and Gallionella groups represent ecotypes dependent on a supply of ferrous iron as the main oxidizable substrate (Winogradsky, 1888*b*). All the subsequent work on the distribution of these organisms has supported the contention that they occur only where reduced iron compounds are continuously available. Nevertheless, the natural environment has not yet been characterized accurately enough to permit further inferences, and the question whether they represent potential chemo-autotrophs is still an open one.

As has been indicated, the most serious difficulty in drawing conclusions from ecological relations is that a satisfactory interpretation must be based on a knowledge of the conditions leading up to the appearance of a particular organism. In this connexion I cannot refrain from mentioning some cogent remarks, made a few years ago at a meeting of American bacteriologists. Eklund, the director of a group of scientists studying the factors responsible for the outbreak of the dreaded poliomyelitis disease, stated that in his opinion the programme is greatly handicapped by the fact that the group is summoned to a particular locality at a time when the disease is already in evidence. It is, unfortunately, not yet possible to predict when and where an epidemic may strike. This precludes the possibility of making on-the-spot observations prior to the appearance of the first cases. It must, of course, be realized that the alternative to the present approach would be to establish a laboratory for polio research at a more or less arbitrarily selected place where the group could assemble data on environmental conditions, on the off-chance that at some time an outbreak might occur there. Obviously, such an approach, though theoretically logical, would render the work most unsatisfactory for the investigators, because of the complete uncertainty of an outbreak occurring, and the establishment of such an institution cannot be particularly appealing to those who must furnish the funds.

These considerations show that the problems of natural selection and the factors governing it can be studied most satisfactorily if one can establish regularities in the appearance of a particular organism in a more or less reproducible environment. In that event one can determine the progressive changes in the latter before and after the appearance of the organism in question. It is not always necessary that the milieu be characterized in great detail; sometimes the factors responsible for the emergence of a special type of microbe can be surmised from observations on highly complex systems. A special case in point is furnished by the ecology of the previously mentioned colourless sulphur bacterium, Thiovulum. It is generally possible to obtain abundant cultures of this micro-organism by mixing a quantity of the marine green alga. Ulva, with some marine mud, placing the mixture in the bottom of a jar, filling this up with sea water, and incubating in darkness. Especially if a trickle of fresh sea water, entering near the bottom of the container, is constantly run in, Thiovulum here finds the ideal conditions for its growth, which is manifested by the appearance of a veil, often of quite irregular shape. Superficially, it suggests a typical stationary condition. Microscopic examination shows, however, that the veil is composed of a vast multitude of extremely motile ovoid microbes, stuffed with sulphur globules. The peculiar distribution appears to be the result of tactic movements by which the organisms maintain themselves in a near optimal situation. Further evidence to this effect is furnished by the observation that the shape and position of the veil is subject to continuous, though often small, alterations, and by the as yet unpublished studies of Dr Wijler. Guided by the assumption that, like other typical sulphur bacteria, Thiovulum grows only when supplied with hydrogen sulphide, which compound is produced during the decomposition of Ulva, he attempted to subculture Thiovulum under better defined conditions. This attempt has been crowned with success; it can be done by introducing a portion of the veil from a crude culture into a container filled with sea water, at intervals adding small amounts of hydrogen sulphide, and maintaining a continuous, slow current of fresh sea water, so that the jar is kept completely filled and overflowing.

These experiments have revealed a number of interesting aspects. In the first place, they have shown that physiologically Thiovulum is a typical sulphur bacterium. If addition of a fresh supply of hydrogen sulphide is postponed too long, the cells gradually lose their characteristic inclusion bodies, and soon afterwards perish. A timely supplementation with hydrogen sulphide causes the rapid reappearance of internal sulphur globules, as in Winogradsky's experiments with Beggiatoa. Ecologically more important is the fact that following a thorough stirring of the culture replenished with hydrogen sulphide, causing a more or less homogeneous dispersal of the organisms, a characteristic veil is re-formed within a matter of minutes, consistent with observations on the rate of movement of the cells, which is several hundred microns per second, and that the highest cell concentrations are found near the inflow of the fresh sea water. Another significant observation is that in a subculture not continuously flushed with sea water a surface film of other bacteria, probably representatives of Thiobacillus, develops. When this film forms, Thiovulum disappears.

It seems reasonable to infer from these results that the optimum conditions for growth of Thiovulum include a delicate balance between the oxygen tension and the hydrogen sulphide concentration of the environment. Deviations therefrom lead to overgrowth by other (sulphur) bacteria whose generation time is considerably shorter. In liquid cultures with a continuous flow of sea water Thiovulum can accumulate by chemotaxis in the most favourable regions; in stationary cultures the diffusion of oxygen into the medium is precluded by the bacterial surface film. It also seems possible that Thiovulum, one of the least-known sulphur bacteria, though described by Warming in 1875 and renamed by Hinze in 1913, may soon become available in pure culture as a result of an extension of Wijler's studies. In that case more detailed physiological experiments will become feasible, and these are necessary for an ultimate interpretation of the process of natural selection.

Nevertheless, however useful 'pure cultures' have been and will continue to be in microbiological work, they can no longer be considered as the eminently constant material which they once seemed to represent. Even in pure cultures variation and natural selection continuously take place, as demonstrated by the often disturbing fact that after a series of transfers to fresh media the resultant crop frequently appears to contain more than one type. The interpretation of this phenomenon has undergone many changes in the course of time, and it is the great merit of W. Braun (Braun, 1947) to have recognized that it can best be ascribed to the continuous appearance of mutants successfully competing with the parent type. We are still far from an adequate appreciation of the specific factors that govern such competition. It is true that Ryan and collaborators (Braun, 1953) have shown the existence of small but significant differences in generation time of wild-type and nutritionally-deficient mutants in favour of the latter, so that at least one of the mechanisms operative in their establishment can be appreciated. Nevertheless, not all mutants are or need be nutritionally deficient, and the highly artificial complex culture media so often used for the maintenance of pure cultures of micro-organisms hamper a more detailed analysis of the selective mechanism. Hence the main emphasis in what follows is concerned with ecological experiments in which numerous different types of microbes participate.

It was especially the work of Winogradsky on a restricted scale (Winogradsky, 1949), and of Beijerinck (Beijerinck, 1921) on a far more general basis, that has made the microbiologist familiar with the possibility of using a direct ecological approach to microbial ecology, by means of the elective, selective, or enrichment culture technique. Here a chemically and physically well-defined environment is chosen; it is inoculated with a sufficient amount of some naturally occurring material to justify the expectation that representatives of most microbial types are thus introduced; and regular observations are made on the microflora and microfauna that develop in the course of time. The outcome of the experiment indicates what organisms come to the fore; these are the ones that are obviously the 'most fit' to survive in competition with others. Such experiments are difficult to perform with larger plants and animals because of the need of large areas, the virtual impossibility of establishing uniform conditions therein, and the long time spans involved. In contrast, the microbes, with sizes of the order of magnitude of microns, permit the use of relatively small culture vessels; and the short generation times, usually measured in hours or fractions thereof, ensure the development of the 'fit' in numbers adequate for further study, often reaching densities of 10^9 individuals per ml. in the course of some days. Also the selection of an appropriate inoculum is simple, because experience has shown that an enormous variety of microbial types is present in a few grams of soil or mud, or in a few ml. of water from stagnant pools and ditches.

It is clear that every microbe in the inoculum capable of development in the environment supplied by the experimenter will multiply. In many cases the organisms that gradually compose the predominant population of such cultures are those whose minimum requirements are fulfilled. For an illustration we may once more draw upon the experience with the sulphur bacteria.

Characterized as autotrophic organisms that oxidize inorganic sulphur compounds such as hydrogen sulphide, they can grow in mineral media. The coloured, photosynthetic, representatives produce an oxidant photochemically; hence they do not require an external oxidizing agent, and can grow anaerobically when properly illuminated. The minimum requirements thus comprise an inorganic, sulphide-containing medium, devoid of an oxidant. And in such media only the coloured sulphur bacteria develop.

It is interesting to contemplate the fact that the environment must contain a special ingredient, hydrogen sulphide, over and above those needed for the growth of algae. Nevertheless, algae do not develop in the crude cultures until the sulphide has been utilized by the sulphur bacteria. The reason for this is that the necessary supplement is inhibitory to algal growth. Diatoms and green algae especially are very sensitive to sulphide; they are usually killed soon after introduction into the elective medium. But the blue-green algae, though much more resistant to the poisonous substance, are also inhibited as long as sulphide is still present. Thus the sulphur bacteria initially outgrow the Cyanophyceae, in spite of the fact that the minimum requirements of the latter are fewer than those of the former.

In this particular case another selective influence may also be introduced. This is the wavelength of the radiant energy. Photosynthesis of the pigmented sulphur bacteria, as of green plants, is accomplished with the aid of chlorophyllous compounds. But the absorption characteristics of the chlorophylls of the green and purple sulphur bacteria are both mutually different, and different from those of the algal chlorophylls; the bacteria display their maximum absorption in the infra-red region where the plant chlorophylls no longer absorb. It is therefore possible to supply cultures with radiant energy of wavelengths not effective for green plant photosynthesis without preventing the growth of the photosynthetic sulphur bacteria. That natural selection is often accomplished through this mechanism is shown by the fact that one may frequently observe mass developments of coloured sulphur bacteria underneath a practically solid layer of algae. And it is feasible to induce the preferential development of green or of purple sulphur bacteria at will by exposing elective cultures to appropriate regions of the spectrum.

The addition of yet another ingredient to the mineral sulphide medium in the form of an oxidizing agent permits the growth of colourless sulphur bacteria. These can grow in darkness where development of photosynthetic organisms is eliminated. The ability of *Thiobacillus denitrificans* to effect an oxidation of reduced sulphur compounds with nitrate as the sole oxidant permits this organism to grow anaerobically in nitrate-containing media; the other colourless sulphur bacteria appear to require free oxygen.

Another illustration of the operation of natural selection through the minimum-requirement mechanism is provided by the microbes that can use molecular nitrogen for the synthesis of proteins and other nitrogenous cell constituents. Long ago Beijerinck found that in strictly mineral media, free of nitrogenous compounds other than N_2, blue-green algae usually develop in illuminated cultures inoculated with garden soil, water, or mud. This ecological observation led him to attribute to these organisms the ability to fix nitrogen. Beijerinck's conclusion was not generally accepted until 1928, when Drewes and Allison independently established this property beyond a doubt with pure cultures.

Large-scale natural selection by the absence of nitrogen compounds occurs around some of the hot springs in Yellowstone Park. Even in regions where the water is no longer warm, only blue-green algae are encountered in abundance. Inoculation of samples of this water with garden soil and incubation of the cultures in the light at room temperature also yields only blue-green algae, whereas the addition of nitrate or ammonium salts to the samples, leads to the development of green algae and diatoms, whose minimum requirements include the need for such substances.

If these observations, made in 1929, had been critically integrated with the noted presence of purple sulphur bacteria in Yellowstone hot springs, it should have been possible to infer the nitrogen-fixing ability of the last-mentioned organisms at that time. The fact that they possess this property was not discovered, however, until quite recently, and as a result of very different observations (Kamen & Gest, 1949; Lindstrom, Tove & Wilson, 1950).

Media devoid of combined nitrogen can also provide cultures of non-photosynthetic microbes capable of fixing nitrogen. For this purpose a supply of an energy-yielding substrate is required. The anaerobic *Clostridium pasteurianum* thus comes to the fore in sugar media incubated in the absence of oxygen; in aerobic cultures representatives of Azotobacter and Beijerinckia form the predominant microflora when the medium is supplied with any one of a number of simple organic substances. Theoretically, nitrogen-fixing bacteria that can live as chemo-autotrophs might exist, notably among the hydrogen-oxidizing types. So far no aerobic hydrogen-oxidizing bacterium with this property has been discovered; but Sisler & ZoBell have shown that *Desulfovibrio desulfuricans*, which can oxidize hydrogen with the concomitant reduction of sulphate to sulphide (Butlin & Adams, 1947; Senez & Volcani, 1951), may, in fact, fix molecular nitrogen (Sisler & ZoBell, 1951).

By the use of elective cultures it is thus possible to prevent the growth of a variety of microbial types present in the inoculum through the use of media of appropriate composition. Nevertheless, even though the principles and execution of this direct ecological approach are quite simple, the results of such experiments are not always easy to interpret. Difficulties are sometimes encountered; they stem, in part at least, from two sources. In the first place, it must always be realized that one introduces with the inoculum a certain amount of foreign material, and this inevitably modifies the environment. In the second place, the development of one or more organisms also results in a gradual modification of the environment, so that the altered medium may become more favourable for the development of other creatures.

The former difficulty can usually be overcome by the use of repeated transfers. In this manner the foreign material is eventually eliminated through dilution, and a more clear-cut relationship can thus be established between a defined environment and the microbes that preferentially develop therein. Occasionally this very procedure may, however, lead to results that are at first sight unexpected and puzzling. A good example of such a situation is furnished by recent investigations of Verhoeven and collaborators. In elective cultures for organisms that can oxidize hydrogen with nitrate in the absence of oxygen, a mineral medium was used and inoculated with garden soil. The first crude culture showed a copious development of *Micrococcus denitrificans*; but growth no longer occurred in transfers. Later it was found that for anaerobic development at the expense of hydrogen and nitrate *M. denitrificans* requires one or more growth factors. These can be provided by a trace of yeast autolysate; in the first culture they were evidently supplied by the soil sample used as inoculum (Verhoeven, Koster & van Nievelt, 1954). A very similar observation was made by Doudoroff in unpublished studies on aerobic hydrogen-oxidizing bacteria. The first crude cultures often contained an abundance of special types that could be perpetuated only in media supplemented with a trace of yeast extract. Another illustration is provided by the fact that elective cultures of Azotobacter tend to diminish in vigour as a result of repeated transfers because for growth in nitrogen-free media the organisms require molybdenum and the soil inoculum supplements the sometimes inadequate molybdenum content of the solution.

A notable instance of an influence exerted by the growth of accompanying micro-organisms has recently been contributed by studies on the obligatorily chemo-autotrophic *Thiobacillus denitrificans.* This organism, discovered more than 50 years ago by Nathansohn and Beijerinck in anaerobically incubated mineral media containing thiosulphate or sulphur as oxidizable substrate and nitrate as the sole oxidant, appears to lose its activity in pure culture. A fairly recent attempt to re-isolate and study the bacterium (Syderius, 1946) was completely unsuccessful; the elective cultures contained so vast a preponderance of heterotrophic denitrifiers that only these bacteria were found on agar plates, a situation which persisted throughout repeated transfers. This is surprising because the composition of the medium is such that it would hardly be expected to support abundant growth of heterotrophs. Syderius suggested that the latter developed at the expense of organic substances synthesized by *T. denitrificans*; but, since this would require a production of organic matter in such profusion that the heterotrophs could outgrow the chemo-autotroph, the interpretation appeared rather improbable.

A different and more satisfactory explanation of the situation has come from the recent studies of the Baalsruds (Baalsrud & Baalsrud, 1954). They established that *Thiobacillus denitrificans* cannot grow at the expense of nitrate nitrogen which the organism readily and quantitatively reduces to molecular nitrogen. Hence it is understandable that nitrogenous substances, other than nitrate, are required for the synthesis of cell material. Indeed, the addition of an ammonium salt to the culture medium results in the copious growth of the denitrifying thiobacilli. Under these conditions the ratio of autotrophic to heterotrophic bacteria in the crude cultures is radically reversed, and the isolation of *T. denitrificans* presents no difficulties. From these observations it appears far more probable that in the cultures containing only nitrate as a nitrogen source growth of the thiobacilli is conditioned by the formation of NH_3 or some other reduced nitrogenous substance through the activities of heterotrophs. This implies that the latter must be present in reasonably large numbers to permit the growth of but a few of the autotrophic bacteria.

It is evident that one must be aware of complications such as those discussed above. Nevertheless, in many studies with elective cultures they are not likely to be encountered. As a rule, repeated transfers lead to cultures that become progressively more homogeneous—at least during their early phases. Later the situation usually becomes more involved because the development of the organisms primarily selected for by the environment causes changes in the medium. This, in turn, often leads to a more or less abundant growth of organisms that are particularly adapted to, and hence will be selected for, by the new conditions. A good example of such a situation is the appearance of large numbers of various protozoa in elective cultures of Azotobacter in nitrogen-free media, especially when the initial concentration of the oxidizable substrate has been low. This is obviously a typical case of a secondary development; it is improbable that anyone would consider the presence of amoebae and ciliates in the cultures as evidence that these organisms use molecular nitrogen as their sole or even main nitrogen source. And microscopic observation of the protozoan behaviour reveals that they have developed because they can feed on the nitrogen-fixing bacteria which form the primary population of the culture. In general, it is not difficult to distinguish the later stages from the primary ones.

Occasionally, elective cultures can be established in which secondary developments do not occur. The best-known is that of anaerobic cultures of lactic acid bacteria in sugar-containing media. These organisms seem to sterilize the medium through the production of lactic acid in concentrations higher than those tolerated by any other microbe—with the exception of *Zymosarcina ventriculi*. The lactic acid bacteria are, consequently, the natural agents through whose activities various sugar-containing foodstuffs can be preserved by anaerobic storage. These organisms are among the most fastidious of bacteria; their nutritional requirements include a variety of B-vitamins and several amino acids. Media of a composition satisfactory for their growth are, of course, more than adequate for the development of many other and less exacting microbes. Nevertheless, the fact is that once again those microbes come to the fore whose minimum requirements are fulfilled, i.e. the lactic acid bacteria. And a simple reflexion will show that otherwise these bacteria would long ago have disappeared, whilst only the organisms with relatively simple needs would have survived.

To be sure, the mechanism whereby successful competition is here achieved is rather different from that discussed before. It may also be expected that additional factors, such as the production of substances that are toxic for potential competitors in very low concentration, as in the case of antibiotics, or differences in growth rate, as in the case of Ryan's nutritionally deficient coli mutants, will play a role in the 'struggle for survival' among micro-organisms. Unfortunately, little is known concerning these matters at the present time. For a better understanding of the problem of natural selection in the microbial world it is, of course, necessary to investigate each situation in detail.

It is also important that special attention be paid to some aspects of elective cultures that are sometimes glossed over. It cannot be denied that the use of such cultures has been instrumental in acquiring a better knowledge of those micro-organisms that predominate in a more or less well-defined environment. Furthermore, such cultures have often supplied the investigator with ideal experimental material for a detailed study of the degradation of a particular substance. By using this as the sole or main substrate in a culture medium, he can readily isolate organisms involved in its decomposition. Conversely, from such elective cultures much has been learned about the functions of various types of organisms in nature. It is a common enough experience that the same kind of bacterium, yeast, mould, or alga can be isolated in different places from similar elective cultures. And it is certainly logical to conclude from such results that also in nature the same type of microbe will be involved in the decomposition of the substrate in question. Sometimes it is thus found that an organism to which hitherto no special role could be ascribed turns up regularly under specified conditions. A striking instance of such a situation is supplied by the recent studies on the bacterial oxidation of pyrimidines. Virtually simultaneously and completely independently, Hayaishi & Kornberg at Bethesda, Wang & Lampen at Cleveland, Lara at Pacific Grove, and Batt at Oxford, isolated the bacteria that develop in mineral media with pyrimidines as the carbon source. In each case the organism responsible for the decomposition of the substrate appeared to be a member of the Corynebacterium-Nocardia group (Hayaishi & Kornberg, 1951; Wang & Lampen, 1952; Lara, 1952; Batt, 1952). Although these bacteria had often been isolated from soil by direct plating on various media, so that their abundant occurrence in soils had been well established, a special ecological niche had not been assigned to them. It is now evident that they are the major, perhaps even the only representatives among the microbes that normally carry out the oxidative degradation of pyrimidines in nature in an otherwise mineral environment.

Experiences such as these are fully in keeping with the once widespread notion that the natural decomposition of many substances always occurs by means of highly specialized types of micro-organisms. Though this concept has its merits, it may also cause confusion if its implications are not clearly recognized. A number of studies on the aerobic decomposition of cellulose aptly illustrate this point. It was early recognized that this polysaccharide can be decomposed by many different fungi; but the bacterial degradation was believed to involve one particular organism, specialized to the point of being unable to use any substrate other than cellulose. When Kellerman, McBeth & Scales announced the isolation in pure culture of a considerable variety of cellulose-decomposing bacteria that could grow quite well in conventional, cellulose-free media, their claim that these organisms were among the causative agents of cellulose degradation in nature was challenged on the basis that the bacteria lacked the requisite degree of specificity. Also the later work of Gray & Chalmers, who had isolated a vibrio that can decompose agar as well as cellulose, was initially interpreted by Winogradsky as indicating that this microbe could not be 'the natural agent' concerned in either process, even if the claim for agar decomposition were sound. Nevertheless, in due course it was convincingly demonstrated that with the aid of elective cultures many different bacterial types can be obtained that are capable of aerobically degrading cellulose, and representatives of the Pseudomonas-Vibrio group, of the aerobic sporeformers, of the actinomycetes, and of the myxobacteria are now known to possess this property. Besides, this is not an isolated instance; in fact the number of processes for which but one single, specific microbe is responsible has dwindled almost to the vanishing point. Nor is there a lack of specificity only in this respect; it is an equally well-established fact that one and the same organism can decompose many different and chemically quite unrelated compounds.

That myxobacteria, pseudomonads, vibrios, actinomycetes, etc., appear in elective cultures with cellulose as substrate must, I believe, be taken to mean that under natural conditions, too, these different types are regularly or occasionally engaged in cellulose degradation. But, whilst their very persistence in nature argues eloquently enough for the recurrence of situations causing their periodic natural selection, it would at present be rash to associate the presence of cellulose in an environment with the mechanism of the particular selection process. The outcome of elective cultures with cellulose as substrate is still wholly unpredictable. Perhaps this must be attributed to the fact that a mineral medium with a piece of filter-paper or cotton-wool, and inoculated with a few grams of soil, is anything but a homogeneous system. Thus the fortuitous presence on a soil particle in contact with a cellulose fibre of large numbers of an organism potentially capable of decomposing the polysaccharide would no doubt cause a localized development of this type, even though the factors normally causing its selection in nature may have little or no connexion with the presence of cellulose.

A comparable situation is presented by the outcome of parallel experiments in which, for example, acetate decomposition is studied under somewhat different conditions. On the one hand, a mineral solution with the substrate is inoculated with, say, 100 mg. of a soil sample; on the other hand, an equivalent quantity of soil is distributed over a culture plate containing the same medium solidified with agar or silica gel. After incubation for an equal length of time the microflora found in the two cultures is strikingly different. The liquid culture generally contains fluorescent pseudomonads to the exclusion of all other types; the plate culture is found covered with colonies of a great diversity of moulds and bacteria, with protozoa feeding on the other micro-organisms.

Such results seem to justify some deductions of importance for the problem of natural selection and the function of specific micro-organisms in nature. The outcome of the liquid culture suggests that the fluorescent pseudomonads are the effective agents for the mineralization of acetate. And no doubt they are. Yet, this conclusion seems contradicted by the cultures on agar, where such organisms are rarely encountered. This is in line with the common experience that pseudomonads do not constitute a quantitatively significant proportion of the soil microflora. The obvious answer to the dilemma thus created is that a completely aqueous environment favours the development of the pseudomonads so that even a single one of these organisms can outgrow competitors, while in soil special conditions cause the natural selection of quite different types for the execution of comparable decomposition processes. A similar experience has been gained from studies on organisms oxidizing hydrocarbons. Söhngen found that on mineral agar plates, inoculated with soil suspensions and exposed to hydrocarbon vapours, colonies of saprophytic mycobacteria appear in abundance (Söhngen, 1913). Later, Haag showed that all mycobacteria tested can oxidize hydrocarbons (Haag, 1927). But in liquid elective cultures, inoculated with the same soil samples, one generally finds pseudomonads as hydrocarbon oxidizers.

It is, therefore, clear that we cannot draw sound conclusions concerning the natural role of microbes from the experience gained with elective cultures unless we restrict our inferences to apply only to rigorously comparable conditions. This has been ably argued by Hungate in connexion with his studies on microbial cellulose decomposition in the rumen of herbivorous animals (Hungate, 1950). Inoculation of a mineral, cellulose-containing medium with rumen fluid, and incubation under anaerobic conditions may yield cultures of cellulose-fermenting spore-formers. But this result merely shows that the inoculum contained such organisms, and not that they fulfil an important function in the rumen, where they may be present in very small numbers. After all, the rumen fluid is very different from a simple mineral solution, and it is only to be expected that in this environment other anaerobic cellulose-fermenting organisms will be selected for. We shall have to learn to interpret the normal occurrence of certain microbes in particular environments in terms that take account of special factors not ordinarily considered. We may find that the common association of pseudomonads, vibrios, spirilla, ciliates, and other highly motile organisms with bodies of water, and of microbes devoid of motility or exhibiting gliding movements, such as myxobacteria, blue green algae and amoebae, with soils or other largely particulate or solid media is the result of natural selection mechanisms that involve more than the mere provision of nutrient materials. It is probable that adsorption phenomena play an important role in creating or maintaining concentration gradients and ionic ratios.

That concentration of nutrients *per se* can influence the results is shown, for example, by experiments on the growth of freshwater algae in liquid elective cultures for which the same mixture of inorganic salts is used at different levels of concentration. In media with high concentrations unicellular green algae predominate; the lower the concentration, the greater is the proportion of filamentous forms developing from comparable inocula. The effect of special ionic ratios is evident from the fact that in solutions with a high magnesium content *Stichococcus bacillarus* tends to outgrow other algae. These illustrations may suffice to indicate a fruitful field for further investigations.

There are many microbes whose 'natural' functions are as yet unknown since they have not been encountered in elective cultures. A good example are the micrococci and sarcinae, so commonly found as aerial contaminants on agar plates. Here, some additional ecological data may help us better to understand the selective processes whereby these organisms manage to persist in nature. It seems to me suggestive that they are abundant on human skin, on cheese rinds, on sausage casings, and even in brine. In view of the complex nutrient requirements of the representatives studied to date, it may well be that they can outgrow potential competitors only in environments where the latter are inhibited by high salt concentrations.*

A curious representative of this group is the sporeforming, motile *Sporosarcina ureae*, first isolated by Beijerinck from liquid elective cultures in urea-containing media. Numerous attempts to re-isolate this organism by similar methods have failed completely. Nevertheless, it appears to be normally present in soil and can be isolated therefrom by direct plating on appropriate agar media, as shown by the extensive studies of Gibson, who also showed that in liquid cultures it is rapidly overgrown by urea-decomposing bacilli (Gibson, 1935). It should be interesting to test the effect of salt concentrations on the outcome of elective cultures.

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* Dr B. A. D. Stocker has called my attention to a publication by Maitland and Martyn (Maitland & Martyn, 1948) which contains pertinent observations and cites earlier work that is fully consistent with this deduction.

The distribution of aerobic sporeforming bacteria in general presents a similar problem. Not a single member of this group is thus far known to thrive in media unsuitable for growth of non-sporeforming organisms. This suggests that the successful competition of the sporeformers is based on factors other than merely nutritional, and the conclusion lies at hand that these are associated with the formation of the highly resistant endospores. Thus they can survive under conditions that cause the destruction of non-sporeforming competitors. The trick of obtaining elective cultures of sporeformers by the use of pasteurized inocula is, of course, based on this principle.

It is true that our knowledge of the natural function of many micro-organisms is still fragmentary. But once an elective culture method for a particular microbe is available, it may be safely concluded that this organism will also be found in nature under conditions corresponding in detail to those of the culture, and that it will then carry out the same transformations.

Thus the elective culture techniques still offer great promise for improving our understanding of microbial ecology and of natural selection. Obviously, the choice of simplified environments for such studies also simplifies the interpretation of the results. It must, however, be recognized that in nature the conditions are seldom simple. Hence we must learn to study more carefully the effects of complicating circumstances, so that it may eventually become possible to realize in the laboratory those conditions that cause the appearance, distribution, and persistence of so many types in nature. This will require much imaginative work, and correlation of many kinds of observations. If we learn, for example, that the mushroom *Panus stypticus* is found in Europe as a non-luminous, and in North America as a luminous species; that the two forms are completely interfertile, and probably differ by no more than a single gene character, as shown by Ruth Macrae (1937); then it is clear that this distribution must be the result of natural selection. And one of the tasks of the general microbiologist will be to elucidate the manner in which such selections operate. It is a formidable task. Nevertheless, the microbiologist can take heart from the knowledge that the material he deals with represents life in a relatively simple form and can often be investigated under conditions far more favourable than those needed for similar studies on higher plants and animals, while yet the fundamental principles he may discover by his efforts will be applicable to all living organisms.

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The Action of Streptococcal Amylase in relation to the Synthesis of an Amylosaccharide by Amylolytic Strains of *Streptococcus pyogenes*

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SUMMARY: Streptococcal amylase behaves like α-amylase, causing branch-chained substrates to lose viscosity with minimal release of reducing groups, and to become achromic with iodine. Reducing values equivalent to 50% of the theoretically obtainable maltose were reached after prolonged hydrolysis.

The enzyme was produced in high concentration by amylase-positive streptococci growing in simplified medium containing substrate. The same strains synthesized a starchy cell substance if grown in plasma-enriched medium containing either maltose or glycogen. When extracted by boiling with weak alkali the new product resembled amylose.

The resemblance of streptococcal amylase to α-amylase was previously noted (Crowley, 1950, 1954). This paper describes a quantitative study of (1) a method of producing potent amylolytic cultures, (2) the amylase action, and (3) the production of a starchy cell substance by the same strains of group A streptococci.

METHODS

Strains. The strains of group A streptococci were SF130, type 1 and type 2/Opaque (both originally isolated by Dr F. Griffith), dried cultures of which were received from Dr Rebecca Lancefield. The strain T2/11020, type 2, had been dried in 1951 when freshly isolated, and was received from the Streptococcal Reference Laboratory.

Culture media. Infusion broth was made by the method of Todd & Hewitt (1932). Peptone dialysate medium was prepared with Benger's peptone (Benger Laboratories Ltd., Holmes Chapel, Cheshire). A suspension of the peptone was dialysed against distilled water to obtain a solution containing *c.* 5% of the dialysable substances; to this were added NaCl 4·5 g./l. and sodium β-glycerophosphate 6 g./l. before sterilizing by filtration. Glutinous corn starch 4%, w/v (Amioca; Brown & Polson Ltd, Wellington House, Strand, London, W.C. 2) was dissolved in 0·067 M-phosphate buffer, pH 7·6. This solution was autoclaved and added to the peptone dialysate in the proportion 1 vol. starch solution to 3 vol. peptone solution making a substrate concentration of 1% (v/v) in the final medium.

Measurement of amylase activity. (1) Viscosity reducing power was measured by a modification of the method of Christensen (1945), using Ostwald pipettes with a flow time for distilled water between 11 and 12·5 sec. at 26°. An enzyme sample (0·5 ml.) was mixed with substrate (4·5 ml.), and the flow time recorded at 2 min. intervals. The substrate, either glutinous corn amylopectin (1·5%, w/v), or non-glutinous corn amylopectin (4%, w/v) was made up in 0·067 M-phosphate buffer and adjusted to an initial flow time of 65–75 sec. at 26°. A fall of 1% in the viscosity (relative to water) of glutinous corn starch at pH 7·0, 26°, during 10 min. was taken as equivalent to 1 unit of amylase. If 0·5 ml. of enzyme reduced the viscosity by 10% then 1 ml. of the sample contained 20 units.

(2) Saccharifying activity was measured by the method of Meyer, Fischer & Bernfeld (1947) as modified by Noelting & Bernfeld (1948). Colorimetric readings were made in a Unicam Spectrophotometer (SP 500) at 480 mμ. Purified corn amylopectin (intrinsic viscosity 1·07) was obtained from the Northern Regional Research Laboratory of the U.S. Department of Agriculture, Peoria, Illinois. Mussel glycogen was from a batch specially prepared by Light and Co. Ltd, Colnbrook, Bucks., England. Purified glutinous corn amylopectin was extracted from commercial Amioca.

pH *values* were measured by a direct recording pH meter (Electronic Instruments, Richmond, Surrey, England, Model 23). 5 l. or larger volumes of culture were continuously neutralized during incubation by an automatic pH controller made by Dr P. J. L. Sequeira, Pathology Department, Royal Free Hospital, which will be described elsewhere. The cultures were incubated in bottles, immersed to the neck in a temperature-controlled bath and were agitated continuously. Bacterial growth was measured turbidimetrically in the spectrophotometer at 650 mμ.

RESULTS

The production of amylase in fluid medium

Little or no amylase was found in 16 hr. Todd-Hewitt broth cultures of the strains T 1, T 2/opaque or T 2/11020. Enzyme was found after 24 hr., reached a maximum after 3 or 4 days at 37° and usually decreased thereafter. The maximum amylase activity in the absence of substrate was usually less than three times that at 24 hr. Following the addition of a suitable substrate to the cultures a sharp rise occurred at any time during 7 days incubation at 37°. When the pH was kept at 7·0 for 6–8 hr. after adding substrate, the increase of enzyme was eight- to tenfold or more. During this period there was a three- to fivefold increase of bacterial growth. When the same strains were grown on starch agar and compared with replica cultures on other media, it was found that amylase was produced by certain colony variants (Crowley, 1954). It seemed probable that in selective cultures amylase-producing streptococci would outgrow the rest of the population. This hypothesis was tested by seeding heavy suspensions of streptococci from 24 hr. broth cultures into peptone water containing glutinous corn starch (1%, w/v), when good yields of amylase were obtained. The method was used to produce potent amylolytic cultures and cell-free filtrates which were relatively low in protein nitrogen. In subsequent experiments the medium was prepared with Benger's peptone, which contained dialysable substances supporting better growth than other culture media tested.

Growth and amylase production in simplified medium

The following procedure was adopted. A dried culture was suspended in 2·5 ml. of broth and from this 1 ml. of growing culture was transferred to 10 ml. of broth which was incubated overnight at 37°. Serial subcultures (10% inoculum) were made in 50 ml., 250 ml. and 1 l. of prewarmed broth at 2 hr. intervals, and finally into 5 l. which was incubated overnight at 37°. The streptococci were centrifuged down and the sediment was suspended in 110 ml. phosphate buffer. 100 ml. of the suspension was added to 300 ml. peptone dialysate + starch substrate and incubated in a water bath at 37° for 1·5 hr. The 400 ml. of culture was then added to 1·6 l. of fresh prewarmed medium and incubation continued for 6·5 hr. The pH value was kept between 6·8 and 7·4 by addition of 10% NaOH solution. Culture samples (25 ml.) were removed at hourly intervals, when bacterial growth was measured turbidimetrically and a cell-free filtrate was tested for amylase by the viscosimetric method. Four ml. supernatant were tested for presence of substrate by adding 1 ml. of iodine solution (iodine 5 g., potassium iodide 10 g., in 500 ml. of distilled water).

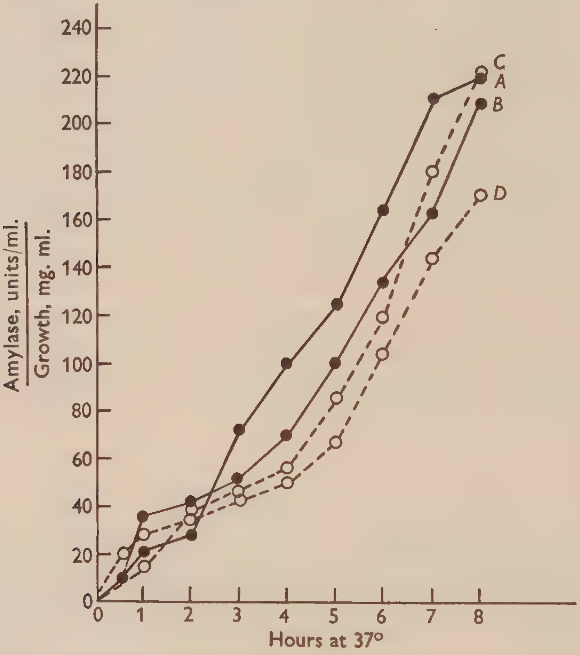

Fig. 1. The ratio of amylase to bacterial growth in the presence of starch substrate. ●——●, type 1 cell suspensions. A, growth increase of 612·3 mg./ml.; and B, growth increase of 497·6 mg./ml. ○ – – – – – ○, type 2 cell suspensions. C, growth increase of 637 mg./ml.; and D, growth increase of 623·4 mg./ml.

The ratio of amylase to bacterial growth during incubation of strains T 1 and T 2/opaque is shown in Fig. 1. The cultures did not usually become completely achromic to iodine for 4·5–5·5 hr. In the later experiments, the substrate was renewed after 4 hr. The bacterial cells were harvested in 110 ml. buffer, and 10 ml. of the suspension was compared with 10 ml. of the initial suspension. The remainder were frozen at −10° and used again. Dilutions of the initial suspension and final suspension were spread on blood agar and starch agar. From the final cultures, 95 % or more of the colonies which grew on blood agar also grew on starch agar and were amylase-positive. For the strain T 1 this was an increase of 25–35 % in comparison with the initial culture, and for the strain T 2/opaque, an increase of 5–15 %.

Production of amylase by the selected population

The thick suspensions of amylase-positive organisms described above yielded amylase in amount equal to or slightly above that of the primary culture when the cells were re-incubated for 5 hr. in fresh peptone + dialysate + substrate medium. When re-incubated a second time the yield was slightly decreased, but after a third re-incubation the yield was halved. It was evident that the amount of amylase obtainable was limited when the organisms were repeatedly subcultured in the simplified medium.

The effect of cell-free filtrates on branched chain amylosaccharides

Action on amylopectin. The viscosity reducing power and saccharifying activity of cell-free filtrates was measured using glutinous corn amylopectin and highly purified non-glutinous corn amylopectin as substrates.

Fig. 2 shows the fall in relative viscosity of glutinous and non-glutinous amylopectin in mixtures incubated at 26°, in which 1 vol. culture filtrate was incubated with 9 vol. substrate. The rate of attack on glutinous amylopectin was greater than on the non-glutinous, though the former was the more viscous substrate. At 37° reduction in viscosity was too rapid to make useful measurements. Glutinous amylopectin showed increased turbidity during the initial stages of the reaction. This was followed by a progressive decrease of turbidity until the substrate became water-clear. The mixtures became achromic to iodine after the viscosity of the substrate was reduced by approximately 50 %.

Saccharifying activity. 1 vol. culture filtrate + 9 vol. substrate were incubated and samples removed at 10 min. intervals during 1 hr. and thereafter at 2, 4, 8 and 18 hr. Reducing values were expressed as percentages of the theoretically obtainable maltose, assuming that the maximum amount of maltose produced by α-amylase from amylopectin was 80 %. Maltose production, initially small, showed a slow linear increase which continued after viscosity reduction had slowed down or ceased. Fig. 3 shows the ratio of maltose liberating activity to viscosity reducing activity when enzyme + substrate mixtures were incubated at 26°.

Action on glycogen. Glycogen (from mussels) when made up in 8 % solution had only about one-third of the viscosity of 4 % non-glutinous amylopectin. The action on glycogen was measured in terms of reducing power, dextrinizing power and decreased turbidity. At substrate concentrations of 4 % or lower, reducing values were of the same order as those obtained with glutinous amylopectin. There was a transient increase of turbidity followed by progressive decrease. The preparation was stained reddish brown by iodine: the mixtures became achromic to iodine during the initial stage of increased turbidity.

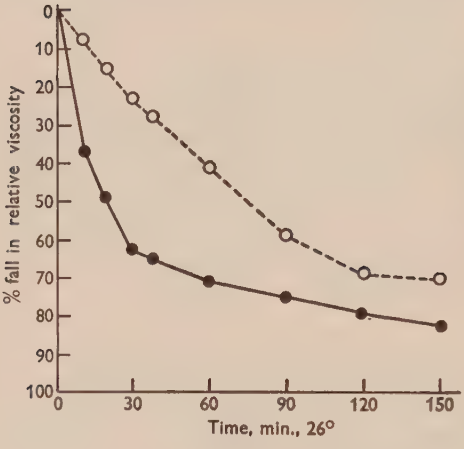

Fig. 2.

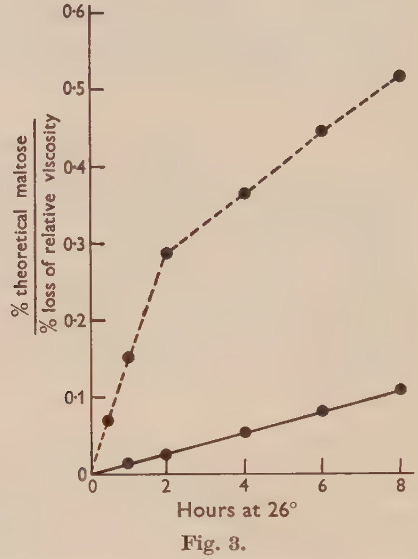

Fig. 3.

Fig. 2. The action of streptococcal amylase on amylopectin. ●——●, the substrate was glutinous corn starch (2 %, w/v) diluted in buffer to have an initial flow time of 70 sec. at 26° at pH 7·0. ○ - - - - - ○, the substrate was purified corn amylopectin (4 %, w/v) in buffer which had an initial flow time of 63 sec. at 26° at pH 7·0. The ratio of enzyme (type 2 filtrate) to substrate was 1 : 9.

Fig. 3. The ratio of saccharifying activity to liquefying activity during hydrolysis of amylopectin by streptococcal amylase.

$$\text{Ratio: } \frac{\text{percentage of theoretical maltose released}}{\text{percentage fall in viscosity relative to water}}.$$

●——●, with enzyme:substrate::1:19.
○ - - - - - ○, as above, with enzyme:substrate::1:1.
The substrate was glutinous corn amylopectin with same type 2 filtrate as in Fig. 2.

Thus the amylase action on branched chain amylosaccharides was characterized by rapid decrease in viscosity, rapid dextrinization and minimal reducing values during the initial stages of the reaction. Reducing sugars increased steadily after the initial reaction had ceased. These properties are typical of α-amylase, but the culture fitrates may have contained other enzymes, and two or more acting together may produce synergic or abnormal effects.

The synthesis of a new amylosaccharide

The amylase action which has been described appeared to be relevant to another finding, namely the production of a starch-like substance by the same strains growing in different cultural conditions. If heavy suspensions of amylolytic organisms were incubated in peptone dialysate medium containing 4% human plasma, and 0·4% of either glycogen or starch, little or no amylase was detected compared with the amount produced in the absence of plasma. Centrifuged sediments from 2 hr. culture samples were, however, stained pale blue by iodine solution, and later samples became dark blue or blue-black. This was due to a starch-like substance produced from maltose, in the absence of starch or glycogen, and the reaction did not therefore require an amylosaccharide as primer. Neither glucose-1-phosphate, glucose, cellobiose, melibiose, saccharose nor lactose was an alternative receptor for maltose.

Heavy suspensions of washed streptococci (strain T 2/11020) were incubated in a maltose-free medium, namely sodium β-glycerophosphate (0·6%, w/v) glycogen (0·2%, v/v) and human plasma (8%, v/v). After incubation for 30 min. at 37°, blue-staining cocci were present in wet films treated with iodine. The starch-like substance was detectable macroscopically after 2–3 hr. incubation, the cultures staining with increasing intensity thereafter. Synthesis therefore took place in the presence either of maltose alone, or of glycogen alone with the help of something in human plasma. The cultures tended to become very acid during growth, and if the pH value was not continuously adjusted to about 7·5 the yield of starchy organisms was poor. The findings suggested that maltose, produced through the amylolytic breakdown of glycogen, was being used to build up a new amylosaccharide when cultural conditions were favourable for the synthesis.

Extraction of the new amylosaccharide

Heavy growth of starch-filled streptococci was obtained by growing the strain T 2/11020 in 5 l. or 8 l. cultures containing β-maltose, 10 g./l. sodium β-glycerophosphate, 25 g./l., Casamino acids, 10 g./l. and human plasma, 10%, v/v. The pH value was kept at 7·5 during incubation for 18 hr. More maltose (as 10% solution) was added after 5–6 hr. The streptococci were harvested and washed four times in phosphate buffer (pH 8). One vol. thick cell suspension in buffer was added to 3 vol. 0·1 N-NaOH held at 100°, at the rate of 50 drops/min., and the mixture was mechanically stirred for 10 min. after adding all the suspension, at a temperature just below boiling-point. After several hours at bench temperature the extract was centrifuged at 3000 r.p.m. The supernatant was adjusted to pH 7·2, any precipitate which formed being removed by centrifugation, and was then chilled for 30 min, and stirred into 4 vol. of cold ethanol. A heavy precipitate separated after standing overnight at 0°. This was washed in ethanol and acetone and dried in air, yielding a white powder which was stained blue by iodine. In addition to the iodine-staining material the crude substance contained peptides, protein and nucleic acid.

Properties of the starch-like substance. The powder was soluble in water at pH 8, being more readily dissolved at 50° or higher temperatures. The iodine-staining material did not diffuse through cellophane; it was precipitated by acetone, methanol, isopropanol, butanol, thymol and cyclohexanol thus resembling the amylose fraction of starch. The complex formed with iodine in neutral solution had a true blue tone; the colour either failed to develop, or faded rapidly, in weakly alkaline solution. Red tones appeared during hydrolysis in acid solution. Aqueous solutions contained occasional round or oval granules like starch granules. The alcoholic precipitates were crystalline, like the addition compounds formed by the amylose fraction of starch with alcohols.

After acid hydrolysis the crude dried substance which contained about 60 % of organic material yielded reducing sugars including glucose. Samples of the amylosaccharide partially purified by precipitation with butanol contained another substance which had a maximum absorption at 260 mμ and was probably nucleic acid. The properties of the amylosaccharide are being further studied.

DISCUSSION

By the degradation of branched chain amylosaccharides like glycogen, amylolytic strains of group A streptococci possess a mechanism for producing maltose which is not a normal constituent of animal tissues. In the presence of something provided by animal plasma, such strains could therefore utilize maltose obtained from glycogen to build up the new amylosaccharide. It has been shown that the synthesis begins within half an hour when amylolytic strains are placed in the appropriate environment. It is clearly possible that such strains may synthesize the starchy substance during passage in the host. Since amyloses readily form all kinds of addition compounds, the presence of the starch might alter the properties of streptococcal products which contribute to pathogenicity. This seems worth consideration with regard to the special types of inflammatory response associated with streptococcal infection in a minority of hosts.

Certain concomitant phenomena in amylolytic strains were previously noted (Crowley, 1954). Many strains freshly isolated from acute infections were untypable by M precipitin tests, and the serotypes most often associated with amylase production were antigenically exceptionally poor for preparation of type specific anti-M sera.

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The Formation of a Starch-like Polysaccharide from Maltose by Strains of *Streptococcus pyogenes*

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SUMMARY: Certain strains of *Streptococcus pyogenes* produced a starch-like substance in the presence of maltose and plasma. Amylolytic strains of group A streptococci produced larger amounts of the substance than other strains of these organisms. The substance was elaborated in the presence of optimal concentrations of plasma, with either starch, glycogen or maltose in the medium. It was not produced when either glucose-1-phosphate, glucose, cellobiose, melibiose, saccharose or lactose was substituted for maltose.

The amylolytic properties of certain strains of group A streptococci were reported by Keogh & Simmonds (1940) and by Crowley (1950, 1954). The latter found that amylase production was a property of certain variants, and that amylase-positive organisms not infrequently lost the property during subculture on medium containing plasma. The effect of serum and plasma on amylase production was further studied, and this paper reports findings related to (1) the effect of plasma on the liberation of amylase, and (2) the formation of a starch-like substance.

METHODS

Strains. The history of six group A strains used is shown in Table 1. All of the strains were originally received from the Streptococcal Reference Laboratory, Public Health Laboratory Service.

Table 1. *The group A streptococci used in the experiments*

Group A strains code no.	Isolation	Animal passages	Serological type		Colony variants
			M	T	
T1/Blue dried culture	By Griffith (SF 130)	Mouse 13	1	1	Matt
T2/44/Rb5 dried culture	By Griffith	Mouse 44, rabbit 4	2	2	Matt
T23/RCC dried culture	By Griffith	—	23	15/12/53	Mucoid
T2/11020 dried culture	S.R.L. 1951	None, fresh isolation	—	2	Matt
T2/Bde514 broth culture	S.R.L. 1954	None, fresh isolation	2	2	Mucoid
T19/714	S.R.L. 1954	None, fresh isolation	19	Not done	Mucoid

S.R.L.—Streptococcal Reference Laboratory.

Culture media. Nutrient agar base (Crowley, 1954) was the minimal medium used. Pooled human plasma (citrated) was sterilized by filtration and added in the concentrations stated in the text. Substrates were prepared in concentrated solution and added as required. Soluble starch (AnalaR, British Drug Houses Ltd., London) and Amioca starch were autoclaved in sodium β-glycerophosphate (2·5%, w/v, solution) at pH 7·8. Amylopectin powder was autoclaved in weighed quantities, the cold paste being made in sterile water and added to boiling glycerophosphate solution. Amylose was dissolved in 2N-NaOH with heat, neutralized with 5N-HCl, and made up to volume as above. Glycogen and sugar solutions were sterilized by filtration. The highly purified corn amylose and corn amylopectin (intrinsic viscosity 1·07) were provided by the Northern Regional Research Laboratory of the U.S. Department of Agriculture, Peoria, Illinois. Glycogen (from mussels) was from a special batch prepared by Messrs Light and Co. Ltd, Colnbrook, Bucks., who also supplied β-maltose and D-glucose-1-phosphate (potassium salt).

Replica cultures were made from imprints on velveteen by the method of Lederberg & Lederberg (1952). Master cultures were selected from tenfold culture dilutions made on blood agar plates, or from dilutions made and spread by the method of Asheshov & Reagy (1951). Amylolytic colonies were detected by exposing plates to iodine vapour.

Gradient plates (Szybalski & Bryson, 1952) were made with sloped layers of 20% plasma agar (15 ml.) and 2% starch agar (15 ml.) as drawn in Fig. 1. The plates were refrigerated for 48 hr. before use.

RESULTS

The effect of plasma on amylase production was tested with four of the group A strains shown in Table 1, which were grown on starch plasma gradient plates. Each of the four strains gave rise to different numbers of amylase-positive clones when blood agar cultures were replicated on starch agar. High plasma or serum concentrations may interfere with the formation of the starch-iodine complex, therefore uninoculated control plates were incubated with the cultures and exposed to iodine vapour at the same time. Four or six culture plates were incubated for each strain, one plate at a time being exposed to iodine vapour after different time intervals. Colonies were visible at the plasma side of the culture (see Fig. 1) after 4–8 hr. incubation, but amylase was not liberated by young cultures, being rarely detectable before 15 hr. at 37°.

The effect of varied plasma/starch concentration on amylase production

The colony variants of strain T 2/11020 in gradient plate culture before and after exposure to iodine are shown in Fig. 1. Mucoid and matt variants growing in high plasma/low starch concentrations did not liberate amylase, whilst organisms growing in low plasma/high starch concentrations produced the enzyme. Colonies in the middle section of the plates were stained blue-black or blue by iodine in cultures of all four amylolytic strains. Mucoid colonies contained faint-staining rings. Matt and opaque colonies were either uniformly stained or ringed either centrally or at the periphery.

The experiments were repeated using gradient plates containing (1) soluble starch, (2) Amioca starch, and it was found that blue-staining colonies were present in cultures of all four amylolytic strains in plasma/substrate concentrations, range 4/0·8% to 8/0·4%. Two amylase-negative strains T 23 and T 19/714 also gave rise to a few blue-staining variants, but only in high substrate/low plasma concentrations. The experiment was then repeated using starch on plasma-layered plates containing (1) constant starch and varied plasma concentration, (2) constant plasma and varied starch concentration. Table 2 shows that amylase production was inhibited at plasma concentrations

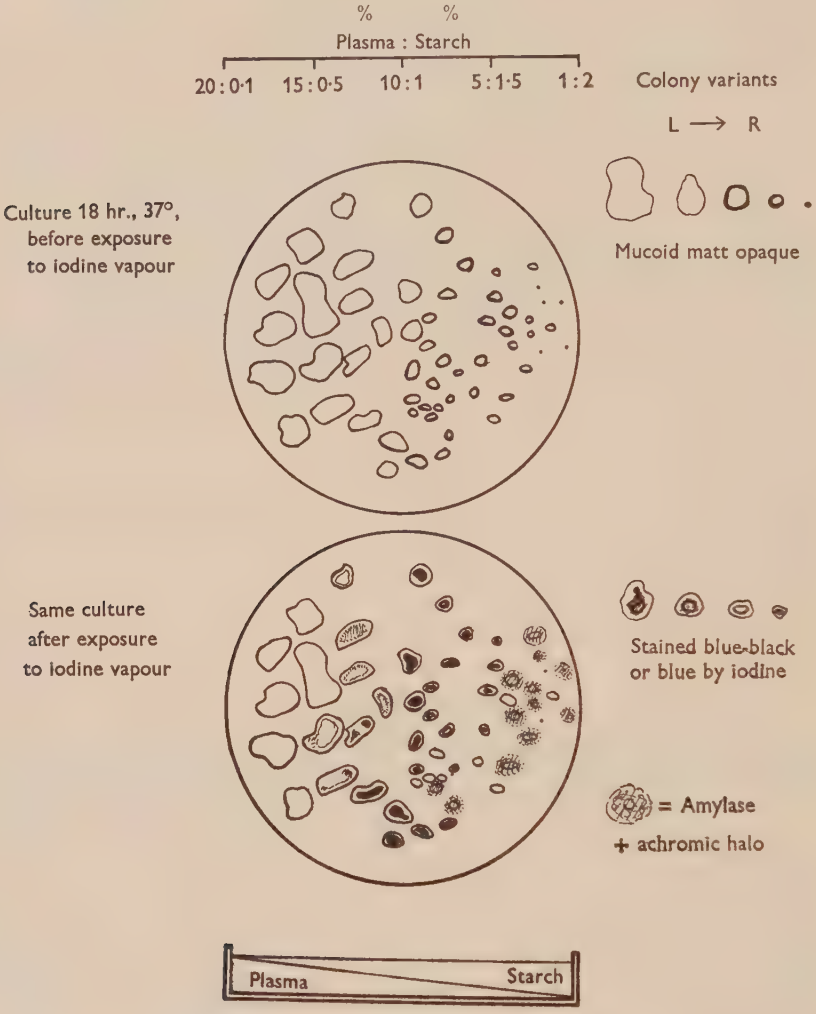
% %
Plasma : Starch
20:0·1 15:0·5 10:1 5:1·5 1:2
Colony variants
L ⟶ R
Culture 18 hr., 37°, before exposure to iodine vapour
Mucoid matt opaque
Same culture after exposure to iodine vapour
Stained blue-black or blue by iodine
= Amylase + achromic halo
Plasma
Starch

Fig. 1. Colony variants of a Type 2 strain on starch-plasma gradient plates.

Table 2. *Amylase-production and formation of starch-like substance at different plasma/starch concentrations*

Amylase + = well-defined achromic zone; ± = hazy zone. Staining + = blue black; ± = faint staining; Ng = no growth.

Strains		Numbers of variants (%) at different starch/plasma concentrations										
	Starch (%) ...	0·1	0·2	0·3	0·4	0·5	1	1	1	1	1	1
	Plasma (%) ...	10	10	10	10	10	10	5	2·5	1·25	0·75	0
T2/44/Rb5. Amylase-positive strain	Amylolytic colonies	0	0	0	0	8 ±	20 ±	10 ±	40	45	37	49
	Stained colonies	37 ±	—	49 ±	> 70	> 70	All	40·8	19	8 ±	0	0
T2/11020. Amylase-positive strain	Amylolytic colonies	0	0	0	0	15 ±	22 ±	42	66	72	90	91
	Stained colonies	0	2·8	6·9	74	75	89	35	30	0	0	0
T23. Amylase-negative strain	Amylolytic colonies	0	0	0	0	0	0	0	0	0	Ng	Ng
	Stained colonies	0	0	0	0	0	0	< 30 ±	> 30 +, ±	30–40 +, ±	Ng	Ng
T2/Bde. Amylase-positive strain	Amylolytic colonies	0	0	0	0	±	±	±	±	±	±	32·5
	Stained colonies	0	0	30–40	30–40	60–70	All	60–70	30–40	30	0	0
T19/714. Amylase-negative strain	Amylolytic colonies	0	0	0	0	0	0	0	0	0	0	0
	Stained colonies	0	0	0	0	< 30	< 30	< 30	30–40	30–40	0	0

of 0·5 % or higher, and that with amylase positive strains there was an inverse relationship between the numbers of amylolytic variants and blue-staining variants in the cultures.

Microscopic appearance of streptococci in blue-staining colony variants

Organisms were suspended in iodine solution (1 vol. 2 % Lugol's iodine and 1 vol. 50 % aqueous glycerol). Wet preparations were examined microscopically by phase-contrast illumination. The chains of cocci from mucoid colonies in overnight cultures were either (1) surrounded by large capsules, or (2) surrounded by small capsules, which varied in size, or (3) non-capsulated. Most of the organisms were unstained or slightly browned by iodine. A single coccus, or paired cocci in chains of 4 to 6 organisms would be stained bright azure blue by iodine. The blue cocci which were non-capsulated were usually larger than the rest, and giant forms were often at the end of a chain of cocci which had small capsules, so that the whole resembled a caterpillar.

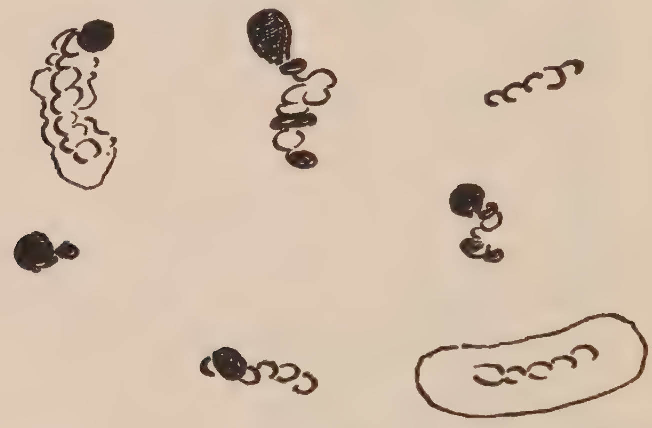

Fig. 2. Starchy streptococci from colony-variants on maltose agar. Shapes seen in iodine-glycerol solution (phase contrast). Black shapes = azure blue cocci.

The formation of starch-like polysaccharide in the presence of other substrates

Three strains were tested for ability to produce the substance in the presence of amylose, amylopectin and glycogen. These preparations were all relatively pure compared with soluble starch or Amioca starch. It was probable, however, that all contained traces of maltose, which was included in the experiment together with glucose, glucose-1-phosphate, saccharose and lactose. The following procedure was adopted: The dried culture was reconstituted in broth, tenfold dilutions of which were inoculated on to blood agar. A suitable culture plate was selected and imprinted on velveteen, after which the master-culture was refrigerated. Replica cultures were made on blood-agar (2 plates) and on a set of polysaccharide or sugar media. One blood agar culture (1a) was replicated after 8–10 hr. at 37° and the second blood agar culture (1A), after 18 hr. incubation. The same culture pattern was therefore reproduced at two phases of growth, early and late. Repeated cultures on to different substrates were made from blood agar replicas of the original culture. After five subcultures, the population was compared with that of the master plate. The transfer error (Lederberg & Lederberg, 1952; Elek & Hilsdon, 1954) was within the range 10–15 % and the range of the numbers of colonies on replica plates is shown in Table 3. In the presence of high plasma concentration, two of the strains formed confluent mucoid growth in which the starchy substance was detectable as a streak or ring near the edge of the growth, as in Fig. 1.

Table 3. *The formation of starch-like substance in the presence of different substrates*

C = confluence, mucoid colonies; ++ = stained blue-black by iodine; + = blue-green; ± = faint staining; Ng = no growth.

		* Numbers of colonies (%) containing starch-like substance					
	Human plasma (%)...	8	8	8	4	2	0
Strains	Substrate (%)...	0·2	0·4	0·8	0·8	0·8	0·8
T1/Blue	Amylose	0	9±	0	0	0	0
	Amylopectin	0	10±	0	0	0	0
	Glycogen	5±	51±	10±	0	0	0
	Maltose	25±	65±	23+	4·5±	0	Ng
	Glucose	0	0	0	0	0	0
	Glucose-1-phosphate	0	0	0	0	0	0
	Saccharose	0	0	0	0	0	Ng
	Lactose	0	0	0	0	0	Ng
T2/11020	Amylose	C/0	C/±	6·5±	0	0	0
	Amylopectin	C/0	C/±	0	0	0	0
	Glycogen	C/±	53+	23·4+	4+	0	0
	Maltose	C/±	52++	27++	6·4+	0	0
	Glucose	C/0	C/0	C/0	0	0	0
	Glucose-1-phosphate	0	0	0	0	0	0
	Saccharose	C/0	C/0	C/0	0	0	0
	Lactose	C/0	C/0	C/0	0	0	0
T23	Amylose	C/0	C/0	C/±	C/±	C/±	Ng
	Amylopectin	C/0	C/0	C/0	C/±	C/±	Ng
	Glycogen	C/0	C/0	C/±	C/±	64+	Ng
	Maltose	C/0	C/0	C/0	50+	93+	Ng
	Glucose-1-phosphate	C/0	C/0	C/0	0	0	Ng
	Saccharose	C/0	C/0	C/0	0	0	Ng
	Lactose	C/0	C/0	C/0	C/0	0	Ng

* The range of the numbers of colonies on the replica plates was as follows: T1/Blue, 92–128; T2/11020, 61–70; T23, 56–72.

Table 3 shows that the streptococci formed the blue-staining substance on maltose plasma agar, but it was not demonstrated in cultures containing saccharose, lactose, cellobiose, melibiose, glucose or glucose-1-phosphate. 'Blue variants' containing 'blue cocci' were demonstrated in the cultures containing glycogen, amylopectin or amylose, which are all composed of maltose chains.

When replica cultures of amylolytic strains were incubated for 2 days or longer, the 'blue substance' in marked colonies was stained with less intensity, and had frequently disappeared. This was attributed to amylase action. It seemed possible that, when the organisms had used up accessory growth factors necessary for the building up and conservation of the starch-like substance coating the cells, it became substrate for amylase. The blue iodine reaction is generally regarded as specific for starch complexes and amyloses. It seemed justifiable to conclude that the cells were coated with an amylose-like polysaccharide.

The production of polysaccharide in the presence of various growth-promoting factors

The results in Table 3 show that the starch-like polysaccharide was not produced by amylolytic strains from maltose in the absence of plasma. The amylase-negative T23 strain, which did not grow on maltose agar prepared with Hartley digest broth (Mackie & McCartney, 1953), was cultured on nutrient agar prepared with broth infused according to the original method of Todd & Hewitt (1932), without added peptone. Growth occurred on this medium when it was enriched with any of the substrates used in the previous experiments, though growth with amylose was very poor. Approximately 30 % of the colonies contained the polysaccharide when either maltose, glycogen or starch components were added to the agar. This was about the same proportion as produced polysaccharide in the presence of plasma. On Todd-Hewitt agar the amylolytic strains produced amylase, and failed to produce the polysaccharide from maltose, glycogen or starch components. Synthesis of the polysaccharide occurred in the presence of bovine plasma, rabbit plasma, horse serum and human serum, but not with bovine albumin, human gamma globulin and human albumin.

Inhibition by monoiodoacetic acid

Replica cultures were made on maltose plasma agar containing different concentrations of monoiodoacetic acid. Before exposure to iodine, 18 hr. growth was replicated on control plates without inhibitor. The production of polysaccharide was completely inhibited by 0·00001 M-iodoacetate. The control plates and control subcultures showed no inhibition. In a similar experiment the formation of polysaccharide was not inhibited by phlorizin (0·001–0·005 M).

Formation of polysaccharide by representative group A strains

Fifty strains of group A streptococci of representative serological types were grown on ox-heart infusion agar containing Amioca starch (0·8 %). With the exception of glycogen, Amioca starch was the most sensitive substrate for detecting amylolytic activity. After 48 hr. incubation at 37°, weak amylase production was sometimes detectable underneath colonies or plaques of growth if the organisms were washed off the medium before exposing cultures to iodine. Fourteen strains were strongly amylase-positive after 48 hr. and twenty-six were amylase-negative. The same strains were then grown on agar containing (1) low substrate/high plasma concentration, (2) high substrate/low plasma concentration. Cultures were exposed to iodine after 18 hr. at 37°. Table 4 shows that most strains which produced the polysaccharide were amylase-positive. Strongly positive strains required low substrate/high plasma medium for producing the polysaccharide but weakly positive strains required high substrate/low plasma medium. These observations suggest that, whilst maltose was the starter for the synthesis of the polysaccharide, the amount of the substance formed varied directly with the amylolytic properties of the strains.

Table 4. *Polysaccharide production by strains in a series of representative group A serotypes*

Polysaccharide production + + +, + + = well-stained colonies; +, ± = faint staining. Serotypes which formed polysaccharide were members of types 1, 2, 4, 12, 13, 14, 19, 22, 25 and 6.

		Production of amylose-like polysaccharide	
Group A strains	Numbers of strains	0·8 % maltose + 2 % plasma	0·4 % maltose + 8 % plasma
Amylase-negative	26	4 + or ±	0
Amylase-negative with 10 % weakly positive variants	10	9 + + + ±	5 + or ±
Amylase-positive	14	6 + ±	14 + + + + + ±
	50	19	19

Qualitative examination of culture plates suggested that strongly amylolytic strains form large amounts of polysaccharide, whereas other strains do not.

DISCUSSION

Bernfeld (1951), summarizing the various modes of biological synthesis of starch-like polysaccharides, distinguished four mechanisms. In two systems the starting substance is glucose-1-phosphate and the transferring enzymes are (1) phosphorylase and isophosphorylase, (2) phosphorylase and *Q* enzyme. Both mechanisms are inhibited by phlorizin. In a third system the starter is saccharose which is transformed into a glycogen-like polysaccharide by amylosucrase, an enzyme found in *Neisseria perflava* by Hehre & Hamilton (1946). In the fourth, an amylomaltase found in the ML strain of *Escherichia coli mutabile* by Monod & Torriani (1950) converts maltose into an amylose-like polysaccharide. The streptococcal mechanism clearly resembles the amylomaltase system. Though a fairly high proportion of strains of *Streptococcus pyogenes* are able to synthesize a starch-like polysaccharide when provided with maltose, certain strains can provide their own starting material by breaking down glycogen (or starch components) to produce a small amount of maltose in the culture.

Culture filtrates of amylolytic streptococci rapidly degrade either amylopectin or glycogen, causing loss of viscosity during the first stage of the reaction, whilst only small amounts of maltose appear in the mixture (Crowley, 1955). Later on the reaction slows down, but maltose slowly increases in amount. In simplified medium with high substrate concentration, the amylase titre steadily rises in the culture, but it has been demonstrated that plasma (or a special factor) in the culture medium prevents the liberation of amylase except in small amounts. The same colony variants are capable of synthesizing an amylosaccharide from maltose, or of breaking down branched chain amylosaccharides. We do not yet know whether both mechanisms are possible in the same cell. We do know that in certain strains the amylase mutation rate is too high to measure by the method of Newcombe (1949) and that the same colony variants readily make large amounts of the starch-like substance from maltose. Crowley (1955) found that the strain T2/11020 also synthesized the amylosaccharide in broth enriched with plasma and maltose at pH 7·5. When extracted from the cells the amylosaccharide resembled amylose rather than glycogen.

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Tricarboxylic Acid Cycle Reactions in the Fungus *Zygorrhynchus moelleri*

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SUMMARY: Whole cells of the fungus *Zygorrhynchus moelleri* can oxidize acetate, *cis*-aconitate, citrate, fumarate, glucose, malate, α-ketoglutarate, pyruvate, and succinate under appropriate conditions. The rates of oxidation of acetate and succinate, in particular, are very high and exceed the rate of glucose oxidation. Diethylmalonate in high concentration inhibits the oxidation of glucose and acetate, but succinate oxidation is not greatly inhibited by malonic acid, even when the malonate concentration is twice the succinate concentration.

By freezing the cells in liquid nitrogen it is possible to investigate many of the individual reactions of the tricarboxylic acid cycle. Among the more important reactions shown were: *cis*-aconitate to citrate; citrate or *cis*-aconitate to α-ketoglutarate; α-ketoglutarate to succinate; succinate to fumarate plus malate; malate to oxalacetate plus pyruvate; and malate or pyruvate to citrate. Acetate was not metabolized. Succinate oxidation was competitively inhibited by malonate. The significance of these results with regard to the operation of the citric acid cycle in this organism is discussed.

Numerous reports have appeared in recent years dealing with the possible operation of a tricarboxylic or dicarboxylic acid cycle as the terminal respiratory mechanism in various micro-organisms (see reviews by Ajl, 1951; Krebs, Gurin & Eggleston, 1952). Among many micro-organisms so examined, bacteria have served as the main source of material, while some work has been performed on unicellular animals (Holland & Humphrey, 1953), and plants (Danforth, 1953), and on yeast (Foulkes, 1951; Krebs *et al.* 1952; Nossal, 1954). Probably, owing to the technical difficulties involved, the filamentous fungi have been relatively neglected from this point of view. Many workers have used indirect methods in order to assess the importance of such cyclic mechanisms in fungal economy (Carson, Foster, Jefferson, Phares & Anthony, 1951; Chughtai, Pearce & Walker, 1950; Halliwell & Walker, 1952; de Stevens, DeBaun & Nord, 1951).

Shu, Funk & Neish (1954), working with *Aspergillus niger* and using tracer techniques with ^{14}C-labelled glucose, have concluded that part of the citric acid formed from glucose arose from recycled C_4-dicarboxylic acid. Garner & Koffler (1951), using the technique of simultaneous adaptation, concluded that the cycle does not exist in *Streptomyces griseus*, and that a conventional succinic dehydrogenase is present in cells grown on succinate but not in those grown on glucose. However, respiratory studies and chemical investigations of individual reactions have tended to support the supposition that a cycle might operate in *Ashbya gossypii* (Mickelson & Schuler, 1953), *Aspergillus niger* (Deffner, 1942), *Blastocladiella emersonii* (Cantino & Hyatt, 1953), *Penicillium chrysogenum* (Casida & Knight, 1954; Hockenhull, Herbert, Walker, Wilkin & Winder, 1954), and *Streptomyces coelicolor* (Cochrane & Peck, 1953). The present work indicates substantially similar conclusions with *Zygorrhynchus moelleri*.

Fungal cell membranes frequently appear to be impermeable to many of the substances regarded as intermediates in the respiratory mechanisms of other organisms, and there have been few reports of attempts to overcome such difficulties by the use of non-viable cell preparations of filamentous fungi.

In the work to be reported here the phycomycete *Zygorrhynchus moelleri* has been examined for the presence of tricarboxylic acid cycle enzymes, using manometric and chemical techniques. The respiratory activities of intact cells and of a non-viable cell preparation towards citric acid cycle substrates have also been examined.

METHODS

Growth and respiration. The cultivation techniques for the organism, *Zygorrhynchus moelleri* (Commonwealth Mycological Institute No. 21994), have been described previously (Moses, 1954). This technique was again followed with the following modifications. Experiments with whole cells were performed with cells starved for 3 hr., unless otherwise stated. The crop from 22 ml. medium was harvested, washed, and resuspended in about 30 ml. phosphate buffer of the molarity and pH indicated for each experiment. 2 ml. suspension was used in each Warburg flask. The flasks were shaken for 3 hr. in order to decrease the level of the endogenous respiration before addition of the substrate. Samples were taken for dry weight and total N determinations.

For experiments with cells treated with liquid nitrogen, 40 ml. spore suspension (obtained from four agar slopes of the organism) were inoculated into four Erlenmeyer flasks (250 ml.), each containing 60 ml. medium. These were shaken in the usual way. The total crop, after washing, was suspended in about 20–25 ml. phosphate buffer of the molarity indicated and at pH 6·5, unless stated otherwise. The very thick suspension so obtained was run slowly from a wide-mouthed pipette, or added with a spoon-shaped spatula, directly into liquid nitrogen, the latter being stirred until the fungus had all been added. The suspension froze into lumps which sank to the bottom of the vessel. (Liquid oxygen has also been used but is less convenient for handling as the frozen lumps float and have a tendency to stick together.) After 30–45 min. the frozen suspension was removed and thawed at 25°. The resulting material was very heterogeneous and difficult to pipette. This was remedied by very gentle homogenization in a Potter homogenizer, the piston being rotated gently by hand and not mechanically. The volume of the resultant uniform suspension was made up to about 30 ml. with buffer, samples were taken for total N determinations, and 2 ml. samples were added to each Warburg flask. In the experiment on malic acid oxidation at two pH values, the cell suspension was made up in water to half the normal volume and 1 ml. dispensed into each Warburg flask; 1 ml. 0·16 M buffer of the required pH value was also added.

Corrections have been made for the CO_2 retained by the buffer (Moses, 1954).

Buffers were prepared by adjusting solutions of KH_2PO_4 to the desired pH value (glass electrode) with KOH or HCl.

Chromatography. (*a*) *Organic acids.* Samples (2 ml.) of the reaction mixture were removed from the Warburg flasks, adjusted to pH 4–4·5 with H_2SO_4 and heated to 70°. The precipitated protein was removed by centrifugation and the supernatant diluted to 50 ml. with water. The acid anions were adsorbed on Amberlite IRA-400 and eluted with 1·0 N-$(NH_4)_2CO_3$ (Bryant & Overell, 1953); all the acids were eluted by the first 20 ml. $(NH_4)_2CO_3$ solution. The eluate was evaporated to dryness over conc. H_2SO_4 *in vacuo* and left in the desiccator until no solid $(NH_4)_2CO_3$ remained. The NH_4 salts of the organic acids were dissolved in water (0·5–1·0 ml.) and a suitable volume (50–100 μl.) applied as a spot to Whatman No. 2 filter-paper. The descending chromatograms were run overnight using a solvent consisting of *tert.*-amyl alcohol (8 parts), chloroform (8 parts), water (8 parts), 98% (v/v) formic acid (3 parts) (L. E. Bentley, personal communication). They were dried in a current of air at room temperature for some hours until all the formic acid had evaporated and were then sprayed with 0·04% (w/v) ethanolic solution of bromcresol green containing a little NH_3. The organic acids appeared as yellow spots on a blue background. Markers of known acids were run as their NH_4 salts: this was important since the R_F of the free acid tended to be rather higher than that of the NH_4 salt, particularly with citric and malic acids. 15 μg. citric acid, as a spot, could be detected in this way. The acids were identified by their R_F values. Citric acid formed from malic acid was also identified by spraying the chromatogram with the Furth-Hermann reagent (Davies, 1953).

(*b*) *Keto-acids.* The method of formation and extraction of keto-acid hydrazones was modified from El Hawary & Thompson (1953). The reaction mixture from the Warburg flask was deproteinized by adding an equal volume of 10% (w/v) metaphosphoric acid and allowing to stand for 1 hr. at room temperature. After centrifugation, the supernatant was treated with an excess of 2:4-dinitrophenylhydrazine in 2 N-HCl for 30 min. at 37°. The hydrazones were extracted into ethyl acetate and then into 10% (w/v) aqueous Na_2CO_3. The latter was acidified with conc. HCl at 0°, the hydrazones again extracted into ethyl acetate and the solution evaporated to dryness in a stream of cold air. The dry hydrazones were dissolved in a little 6% (w/v) aqueous NH_3 and applied to Whatman No. 3 filter-paper as spots or bands. Descending chromatograms were run overnight using *n*-butanol saturated with 6% (w/v) aqueous NH_3 as solvent (Fincham, 1953), the keto-acid hydrazones being identified by their R_F values and by reduction to the corresponding amino acid. Known keto-acid 2:4-dinitrophenylhydrazones were run as markers.

The keto-acid chromatogram was dried in a current of air at room temperature, the spot cut out, and macerated in 6% (w/v) aqueous NH_3. After 1 hr. the suspension was centrifuged, the supernatant acidified with conc. HCl at 0° and extracted with ethyl acetate. The latter was evaporated to dryness in a current of air at room temperature. The hydrazones were dissolved or suspended in about 1 ml. 0·5–1·0 N-CH_3COOH, Adam's catalyst added, and the mixture treated with H_2 at a pressure of 50 lb./sq.in. for 15–30 min. until the yellow colour had disappeared (Towers, Thompson & Steward, 1954). The solution of amino acids was evaporated to dryness *in vacuo* over conc. H_2SO_4. The amino acids were redissolved in a suitable volume of water and applied to Whatman No. 3 or 4 filter-paper for one-dimensional chromatograms using as solvents either 75 % (v/v) phenol, 25 % (v/v) water in an atmosphere of NH_3, or 40 % (v/v) *n*-butanol, 50 % (v/v) water, 10 % (v/v) glacial acetic acid. After drying at 80–90°, and spraying with ninhydrin, the amino acids were identified by their R_F values and by comparison with known markers run on the same chromatogram. Two-dimensional chromatograms were run with phenol-NH_3 in one direction and collidine-lutidine in the other, the amino acids being identified from the map given by Dent (1948); known amino acids were incorporated as markers.

Chemicals. All chemicals were commercial products except oxalacetic acid 2:4-dinitrophenylhydrazone which was a gift from the Biochemistry Department, University College, London. Armour Laboratories' 'Liver Coenzyme Concentrate' was used as a source of coenzyme A and of di- and triphosphopyridinenucleotides.

Acid substrates were used as their Na salts, and solutions were adjusted to a definite pH value before use.

Dry weight and total nitrogen determinations. Dry weights were obtained by filtering portions of cell suspensions through weighed sintered glass crucibles and drying at 105–110° to constant weight. Total N determinations were performed by the standard micro-Kjeldahl technique, using a selenium catalyst during the digestion process which was continued for 8 hr. after the solution had cleared.

RESULTS

Respiration of intact cells

The ability of intact unstarved cells to oxidize tricarboxylic acid cycle intermediates, acetate and glucose was examined. At high pH values (6·8) only glucose and acetate stimulated the rate of oxygen uptake (Table 1). When the pH value was lowered to about 3·4 all the intermediates tested were oxidized (Table 1), though now acetate was inhibitory even to the endogenous respiration (Moses, 1955*b*). The presence of permeability barriers to weak acid substrates is well known in micro-organisms, and attempts have been made to overcome them either by working at low pH values so as to suppress the ionization of the weak acids, or by the use of cell-free extracts. Krebs *et al.* (1952) found, however, that with baker's yeast a low pH value was ineffective in removing the permeability barrier to these acids.

The absolute rates of oxidation of the various substrates by *Zygorrhynchus moelleri* varied considerably from preparation to preparation, although the relative order remained the same. For example, the Q_{O_2} (succinate) has varied from 40 to 105; the values given in Table 1 are typical.

Table 1. *Rates of oxidation of tricarboxylic acid cycle intermediates, glucose, and acetate*

Warburg flasks contained in a total volume of 3·0 ml.: 5·76 mg. dry wt. living cells (0·53 mg. cell N), 0·1 M-K phosphate buffer of the pH indicated, and 0·02 M-substrate. Centre wells contained 0·2 ml. of 15 % (w/v) KOH. Gas phase air; temp. 25°. Final max. rates of O_2 uptake recorded.

Substrate	pH	Q_{O_2}	q_{O_2} (N)
None (endogenous)	3·4	10·4	113·6
cis-Aconitate	3·4	13·8	151·6
Citrate	3·4	28·4	310·8
Fumarate	3·4	53·2	579·8
Glucose	3·4	68·0	742·8
L-Malate	3·4	37·6	409·2
α-Ketoglutarate	3·4	51·4	560·8
Pyruvate	3·4	59·0	644·2
Succinate	3·4	70·4	769·2
None (endogenous)	6·8	16·2	177·2
Acetate	6·8	125·6	1371·6
Glucose	6·8	92·4	1008·0

By the oxidation of small, known, quantities of these acids and measurement of the total oxygen uptake and carbon dioxide evolution in excess of the endogenous respiration, assuming the latter to continue during the respiration of external substrates (Moses, 1955*a*), the degrees of total oxidation and R.Q.'s were determined (Table 2). A lag was observed in the oxidation of all these substrates, particularly citrate, affecting both the oxygen uptake and carbon dioxide output (Fig. 1).

Table 2. *Degree of total oxidation and* R.Q. *for tricarboxylic acid cycle intermediates*

Warburg flasks contained in a total volume of 3·0 ml.: 3·72 mg. dry wt. cells, 0·067 M-phosphate buffer, pH 3·4, and 3 μmoles of each substrate (6 μmoles of DL-malate). Centre wells contained 0·2 ml. of 15 % (w/v) KOH where necessary. Gas phase air; temp. 25°. Values reported are corrected for the endogenous gas exchange.

	Substrate				
	Citrate	Fumarate	Glucose	DL-Malate	Succinate
μmole O_2 absorbed/μmole substrate:	1·52	1·07	2·17	1·40	1·49
μmole CO_2 evolved/μmole substrate:	3·20	2·09	2·41	2·52	2·14
R.Q.:	2·11	1·95	1·11	1·80	1·44
Theoretical R.Q. for complete oxidation:	1·33	1·33	1·00	1·33	1·14
Degree of total oxidation based on CO_2 evolution (%):	53·3	52·5	40·2	63·1	53·6

Total endogenous O_2 uptake during experiment: 10·7 μmole.
Total endogenous CO_2 evolution during experiment: 9·4 μmole.
R.Q. (endogenous) = 0·88.

The action of malonate on the ability of the cells to oxidize succinate was investigated (Table 3). The malonate concentration necessary to produce inhibition was relatively high; the degree of inhibition was maximal immediately after the addition of malonate, decreasing as the experiment proceeded. Even when the malonate concentration was twice that of the succinate the degree of inhibition was small. As subsequent work showed that succinoxidase in this organism is competitively inhibited by malonate in the usual way, it is interesting to note the ability of the cell to protect its enzymic

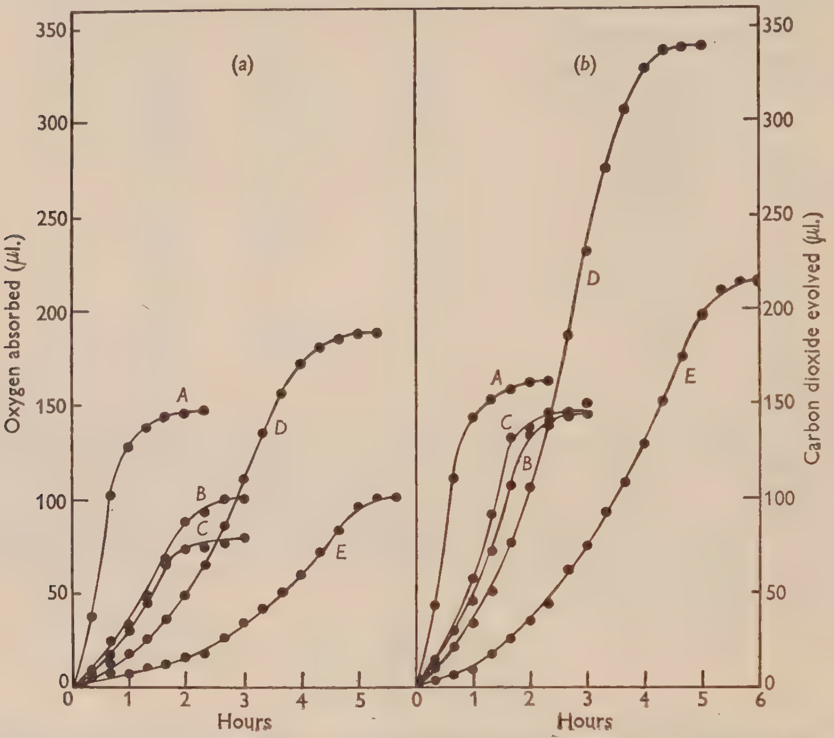

Fig. 1. Gas exchange of intact cells metabolizing various substrates. Experimental details as Table 2. Substrate added at time zero; endogenous gas exchanges deducted. *A*, glucose; *B*, succinate; *C*, fumarate; *D*, DL-malate; *E*, citrate. (*a*) Oxygen uptake; (*b*) carbon dioxide evolution.

Table 3. *Inhibition of succinate oxidation by malonate in whole cells*

Warburg flasks contained in a total volume of 3·0 ml.: 2·8 mg. dry wt. cells, 0·067 M-phosphate buffer, pH 3·4, and, where added, 0·01 M-succinate and malonate as indicated. Centre wells contained 0·2 ml. of 15 % (w/v) KOH. Gas phase air; temp. 25°. Malonate added to the cells simultaneously with the succinate. Endogenous O_2 uptake subtracted.

Malonate concn. (M) ...	0·0	0·02	0·016	0·01	0·006	0·002
$\frac{\text{Succinate concentration.}}{\text{Malonate concentration.}}$	—	0·5	0·625	1·00	1·67	5·00
O_2 uptake (µl.) in 270 min.	642	474	483	740	620	610
Inhibition (%)	—	26·2	24·7	−15·3	3·4	5·3
Final rate of O_2 uptake (µl./hr./flask)	200	180	177	230	200	200
Inhibition (%)	—	10·0	11·5	−15·0	0·0	0·0

Total endogenous O_2 uptake in 270 min.: 268 µl.
Final endogenous rate of O_2 uptake: 30 µl./hr./flask.

apparatus against inhibition even when it seems probable that the malonic acid was able to enter the cells.

Beevers, Goldschmidt & Koffler (1952) have suggested the use of esters of weak acids to overcome permeability barriers at high pH values. As acetate was oxidized by *Zygorrhynchus moelleri* at high pH values only, and malonic acid was active at low pH values only, the action of diethylmalonate on acetate and glucose oxidation was investigated, and compared with the effect of a quantity of ethanol equivalent to that present in the ester. Considerable inhibition could be produced by high concentrations of the ester, though lower concentrations were inactive or even stimulatory. Acetate oxidation was more sensitive to the ester than was glucose oxidation; ethanol was virtually without effect. (Table 4). The latter observation indicates that effects due to the ester may be attributed to the acid moiety of the molecule and not the alcohol.

Table 4. *Inhibition of glucose and acetate oxidation by diethylmalonate*

Warburg flasks contained in a total volume of 3·0 ml.: 4·3 mg. dry wt. cells starved in buffer for 24 hr., 0·067 M-phosphate buffer, pH 6·8, and, where added, 0·022 M-glucose, 0·067 M-acetate, and diethylmalonate and ethanol as indicated below. 0·2 ml. of 15 % (w/v) KOH in centre wells. Gas phase air; temp. 25°. Endogenous values not subtracted.

	Glucose oxidation				Acetate oxidation			
Diethlymalonate concn. (M)	0·00	0·10	0·03	0·01	0·00	0·10	0·03	0·01
O_2 uptake before addition of ester (μl./hr./flask)	165	165	165	165	293	293	293	293
O_2 uptake after addition of ester (μl./hr./flask)	—	6	116	246	—	5	72	264
Inhibition (%)	—	96·4	29·7	−49·1	—	98·3	75·5	9·9
Ethanol concn. (M)	0·00	0·10	0·03		0·00	0·10	0·03	
O_2 uptake before addition of ethanol (μl./hr./flask)	165	165	165		293	293	293	
O_2 uptake after addition of ethanol (μl./hr./flask)	—	175	170		—	260	260	
Inhibition (%)	—	−6·1	−3·0		—	11·3	11·3	

This work with intact cells suggested that whatever systems might be present in the fungus for the oxidation of organic acids, the reactions were very well integrated (as evidenced by the high rates of respiration) and well protected against inhibitors. The prospects of isolating any individual reactions in such cells did not appear to be good, and it was considered desirable to attempt to find a cell-free preparation capable of respiring tricarboxylic acid cycle intermediates similar to those metabolized by whole cells.

Non-viable cell preparations

Various techniques for overcoming the permeability difficulties encountered with whole cells were investigated. The technique of treatment with liquid nitrogen (Lynen & Neciullah, 1939) finally adopted gave preparations as active as any tried and was easy to carry out.

The methods investigated included: drying of the cells *in vacuo* over various desiccants; freeze-drying; acetone powdering; disruption in a high-pressure apparatus (Milner, Lawrence & French, 1950; French & Milner, 1951); disruption in a bacterial press (Hughes, 1951); grinding in a cold mortar with sand and either 0·4 M-sucrose or mannitol, or the alkaline isotonic potassium chloride solution of Potter (1948); treatment with solid carbon dioxide ('dricold') (Krebs *et al.* 1952). Methods involving drying generally resulted in inactive preparations; those involving disruption produced active preparations, though the presses were inconvenient to use; grinding by hand was very tedious and the percentage of cells disrupted quite small even after some 10 min. Grinding in a Potter tissue homogenizer or disruption in a Waring blender were ineffective. 'Dricold' was less effective than liquid nitrogen, probably because the rate of cooling of cells deposited on the 'dricold' in a thin plastic tube was much less than when the cell suspension was added directly to liquid nitrogen. Krebs *et al.* (1952) were able to pack alternate layers of 'dricold' and crumbled commercial baker's yeast obtained from a block. Good contact was thus obtained between the yeast and the 'dricold' and the cooling was consequently rapid. *Zygorrhynchus moelleri* could not be obtained in a similar state so that the contact was inferior and the rate of cooling less.

Attempts were made to obtain a mitochondrial preparation from the organism. After grinding in a cold mortar, the remaining complete cells, cell débris, and sand were centrifuged at 2000 *g*. The supernatant fluid was centrifuged for 15 min. at 10,000 *g*. A pellet was obtained of approximately uniformly sized particles which stained with tetrazolium chloride and Janus Black in the manner of mitochondria. The preparation showed almost no oxidative activity, due perhaps to an unavoidable rise in temperature to 10–12° during centrifugation. As a refrigerated centrifuge was not available the technique was not pursued further.

Cells treated with liquid nitrogen were used in the following experiments in which several individual steps in the tricarboxylic acid cycle were investigated.

Succinate oxidation

Succinate was oxidized with a small evolution of carbon dioxide (Table 5) Chromatography of the products demonstrated the formation of fumarate and malate. Although succinate was oxidized by the preparation more rapidly than any other substrate tested, the rate of oxidation was only about 4% of that by whole cells (cf. Table 1).

Table 5. *Oxidation of succinate by liquid nitrogen-treated cells*

Warburg flasks contained in a total volume of 2·5 ml.: 5·30 mg. cell N, 0·067 M-phosphate buffer, pH 6·5, and, where added, 0·0625 M-succinate. For measurement of the O_2 uptake the centre wells contained 0·2 ml. of 15% (w/v) KOH. Gas phase air; temp. 25°.

		With succinate	
	Endogenous	Uncorrected	Minus endogenous
q_{O_2}(N) (30–90 min.)	4·0	27·9	23·9
q_{CO_2}(N) (30–90 min.)	3·5	5·6	2·1
R.Q.	0·88	0·20	0·09

Effect of malonate on succinate oxidation. Succinate was oxidized in the presence of various concentrations of malonate. Using the formula (Krebs *et al.* 1952):

$$\frac{1-n}{n}\times\frac{[\text{substrate}]}{[\text{inhibitor}]}=K, \quad \text{where} \quad n=\frac{\text{reaction rate with inhibitor}}{\text{reaction rate without inhibitor}},$$

it was found that succinate oxidation is competitively inhibited by malonate. K, the ratio [succinate]/[malonate] giving 50% inhibition (after correction for the blank respiration), was about 12 (Table 6). It is of interest that this value approximates to the value of about 10 for malonate inhibition of succinoxidase in mammalian muscle tissue, and is completely different from the value of about 60 found by Krebs *et al.* (1952) for yeast.

Table 6. *Malonate inhibition of succinate oxidation*

Each flask contained 3·89 mg. cell N. Substrate concn. as indicated. Other conditions as in Table 5. For explanation, see text.

Succinate	Malonate	Succinate concn.	q_{O_2}(N) (30–90 min.)			
$\text{M}\times10^2$	$\text{M}\times10^4$	Malonate concn.	Uncorrected	Minus blank	n	K
0·0	0·0	—	2·8	—	—	—
6·25	0·0	—	21·9	19·0	—	—
6·25	50·0	12·5	12·3	9·5	0·50	12·7
6·25	25·0	25·0	15·4	12·6	0·66	12·8
6·25	12·5	50·0	18·3	15·5	0·82	11·4
6·25	6·25	100·0	19·8	17·0	0·89	12·2
0·0	50·0	—	3·6	0·8	—	—

Average value for $K=12·2$.

Fumarase activity

10 ml. of an aqueous suspension of treated cells was introduced into each of two gas-washing bottles containing 0·5 ml. M-sodium bicarbonate and 4·5 ml. water. The bottles were incubated at 25° and nitrogen containing 5% (v/v) carbon dioxide was continuously bubbled through the suspensions. After 15 min. 15 ml. 0·1 M-sodium fumarate was added to one bottle and 15 ml. water to the other. Samples (5 ml.) were removed from each bottle at 0, 1, 2·5, and 3·5 hr. after the addition of substrate for estimation of malic acid by polarimetric analysis. Each sample was rapidly mixed with 1·25 ml. glacial acetic acid, 6 ml. water, 2·5 ml. M-sodium citrate and 11·25 ml. 29% (w/v) ammonium molybdate. After filtration, readings were taken in a 2 dm. tube using sodium light and the amount of L-malic acid formed was calculated using $(\alpha)_D=+1340°$ for the specific rotation of the malate-molybdate complex in the presence of citrate (Krebs & Eggleston, 1943). The amount of malic acid formed is shown in Table 7. Chromatography of the product confirmed the presence of malic acid.

Decarboxylation and oxidation of α-ketoglutarate

Without the addition of cofactors α-ketoglutarate was not oxidized, though it was decarboxylated. In a system containing treated cells (4·32 mg. cell N), 0·067 M-phosphate buffer, pH 6·5, and 25 μmole α-ketoglutarate in a total

Table 7. *Fumarase activity*

Gas washing bottles contained: 10 ml. treated cell suspension (25·82 mg. cell N), 0·5 ml. M-$NaHCO_3$, 4·5 ml. H_2O, and either 15 ml. 0·1 M-Na fumarate or 15 ml. H_2O. Bottles gassed with 95 % (v/v) N_2 + 5 % (v/v) CO_2. Samples (5 ml.) withdrawn at intervals for estimation of malate.

Time of incubation (hr.)	L-Malic acid formed (μmole)		Conversion of fumarate to malate (%)
	Control	With fumarate	
0	0·0	33·6	2·24
1	9·0	252·9	16·86
2·5	31·3	534·9	35·66
3·5	4·5	702·8	46·85

volume of 2·5 ml., together with 0·2 ml. of 15 % (w/v) potassium hydroxide in the centre wells where necessary, the oxygen uptakes in 4 hr. without and with substrate were 54·3 and 54·5 μl. respectively. The corresponding carbon dioxide evolutions were 61·8 and 171·1 μl.

This pattern of gas exchange suggested decarboxylation of α-ketoglutarate at either the α- or γ-positions to produce succinic semialdehyde (β-aldehydopropionic acid) or α-ketobutyric acid, respectively. Chromatograms of the keto-acid 2:4-dinitrophenylhydrazones prepared from the reaction mixture showed two large yellow areas at R_F 0·47 and 0·36. These were hydrogenated and the resulting amino acids run on two-dimensional chromatograms with phenol-ammonia and collidine-lutidine as solvents. The keto-acid hydrazone spot of R_F 0·36 produced no amino acid and its identity remains obscure. The other substance (R_F = 0·47) was reduced to an amino acid identified as γ-aminobutyric acid. The keto-acid from which this arose was thus succinic semialdehyde; α-ketobutyric acid would be reduced to the amino acid α-aminobutyric acid.

Table 8. *Oxidation of α-ketoglutarate*

Warburg flasks contained in a total volume of 2·5 ml.: 4·99 mg. cell N, 0·1 M-phosphate buffer, pH 6·5, 'liver coenzyme concentrate' 2 mg., ATP 2 mg., methylene blue 0·1 mg., 0·01 M-$MgSO_4$, and, where added, 0·02 M-α-ketoglutarate, 0·005 M-malonate. The centre wells contained 0·2 ml. 15 % (w/v) KOH. Gas phase air; temp. 25°.

	Endogenous	α-Ketoglutarate	Malonate	α-Ketoglutarate + malonate
O_2 uptake (μl.) in 240 min.	200·9	272·3	206·8	241·7
O_2 uptake minus endogenous	—	71·4	5·9	40·8
Malonate inhibition (%):				
(i) endogenous not subtracted	—	—	—	11·2
(ii) endogenous subtracted	—	—	—	42·9

In the presence of methylene blue, 'liver coenzyme concentrate', adenosine triphosphate (ATP) and magnesium sulphate, liquid nitrogen-treated cells oxidize α-ketoglutarate slowly. The rate of oxidation was inhibited by malonate (Table 8). In the presence of malonate some succinate was detected chromatographically. In the absence of malonate no succinate accumulated. (The sample of α-ketoglutarate used contained some succinate as impurity, but this was insufficient to account for the succinate formed during the oxidation of α-ketoglutarate in the presence of malonate.) The demonstration earlier of a malonate-sensitive succinoxidase system in *Zygorrhynchus moelleri* suggests that inhibition of α-ketoglutarate oxidation by malonate is, in effect, an inhibition of the further oxidation of the succinate formed. It is, nevertheless, worth noting that Price (1953) has found that α-ketoglutarate oxidase from pea stems is inhibited by 0·03 M-malonate.

Oxidation of citrate and cis-*aconitate*

Both citric and *cis*-aconitic acids stimulated the uptake of oxygen by the cold-treated cells without the addition of co-factors. The rate of citrate oxidation, however, was slow. In an experiment in which each Warburg flask contained: cell material (7·22 mg. cell N), 0·1 M-phosphate buffer, pH 6·5, and 100 μmole substrate in a total volume of 2·5 ml., with KOH in the centre well, the oxygen uptake in 3 hr. with no substrate, with citrate and with *cis*-aconitate was 78, 121, and 297 μl., respectively.

Attempts to demonstrate aconitase activity polarimetrically by the formation of L-*iso*citric acid from citric acid, in a manner similar to that of Krebs *et al.* (1952), were not successful. It was possible, however, to show the formation of citrate and α-ketoglutarate from *cis*-aconitate. Chromatography of the organic acids produced in the respiration experiment described above showed the presence of citric acid and α-ketoglutaric acid in the flasks containing *cis*-aconitic acid, but no reaction product from the flasks containing citric acid.

By using the more sensitive technique of keto-acid hydrazone chromatography, the qualitative formation of α-ketoglutarate from both citrate and *cis*-aconitate could be shown in the same system. The amount formed was not determined accurately, but the sizes of the spots of α-ketoglutarate 2:4-dinitrophenylhydrazone produced from equal volumes of the reaction mixture showed a greater formation from *cis*-aconitate than from citrate.

The identity of the α-ketoglutarate was confirmed by hydrogenation of the eluted hydrazones and identification of the resulting amino acid as glutamic acid by chromatography.

Oxidation of L-*malic acid*

The oxidation of L-malic acid was examined at pH 6·5 and 9·0, and also at pH 9·0 in the presence of glycine. Eisenberg (1953), investigating L-malate oxidation by dried cells of *Rhodospirillum rubrum*, found that the oxidation had a pH optimum of 9·0 and was inhibited by the oxalacetate formed, or by added oxalacetate. The use of glycine + phosphate buffers at pH 9·0 stimulated the rate of oxidation, due apparently to the decarboxylation of the oxalacetate by the amino group of glycine. Ostern (1933) found that oxalacetate is decarboxylated by the amino group of aniline.

The *Zygorrhynchus moelleri* preparation showed a greater rate of oxidation of L-malate at pH 9·0 than at pH 6·5, but the addition of glycine at pH 9·0 caused no significant stimulation (Table 9).

Table 9. *Oxidation of* L-*malic acid*

Warburg flasks contained in a total volume of 2·5 ml.: 8·27 mg. cell N, 0·08 M-phosphate buffer of the pH indicated below, 'liver coenzyme concentrate' 2 mg., ATP 2 mg., methylene blue 0·1 mg., 0·01 M-$MgSO_4$, and, where indicated, 0·08 M-glycine (pH 9·0) and 0·04 M-L-malate. The centre wells contained 0·2 ml. 15 % (w/v) KOH. Gas phase air; temp. 25°.

	O_2 uptake (μl.) in 180 min.		
Conditions	Endogenous (*a*)	L-Malate (*b*)	(*b*) − (*a*)
pH 6·5	41·6	125·6	84·0
pH 9·0	61·2	185·9	124·7
pH 9·0 + glycine	60·6	190·7	130·1

The products of malic acid oxidation were investigated by chromatography of the keto-acid hydrazones. The experimental conditions were those in Table 9 at pH 6·5 without glycine. An examination was also made of the products accumulating in the presence of 0·02 M-potassium cyanide as a trap for keto-acids. In the presence of cyanide keto-acid 2:4-dinitrophenylhydrazones corresponding to those of authentic samples of pyruvic and oxalacetic acids were obtained, the latter in small quantities only. In the absence of cyanide pyruvate but no oxalacetate was detected. The identity of the pyruvate derivative was confirmed by hydrogenation and comparison of the product with an authentic sample of alanine by chromatography in two solvents. Quantities of the oxalacetate derivative sufficient for positive confirmation of its identity were not obtained: chromatography of the hydrogenated derivative in phenol-ammonia showed a very faint spot corresponding to aspartic acid. Owing to the small amount of material available it was not chromatographed in butanol-acetic acid.

Pyruvate and acetate metabolism

Pyruvate was decarboxylated very rapidly, both aerobically and anaerobically, with the production of acetaldehyde. The latter was identified qualitatively by the production of a violet colour following the addition to the reaction mixture of pyridine followed by sodium nitroprusside. The rate of oxygen uptake with pyruvate was low and, being very much smaller than the rate of carbon dioxide evolution, was difficult to measure accurately. The metabolism of pyruvate and malate together produced a gas exchange less than the sum of the gas exchanges with each substrate separately (Table 10).

Table 10. *Pyruvate, acetate, and* L-*malate oxidation*

Warburg flasks contained in a total volume of 2·5 ml.: 5·78 mg. cell N, 0·05 M-phosphate buffer, pH 6·5, 0·04 M-substrate, and the supplements as in Table 9. Gas phase air; temp. 25°. Endogenous respiration has been deducted.

		Substrate				
	Endogenous	Malate	Pyruvate	Acetate	Malate + pyruvate	Malate + acetate
O_2 uptake (μl.)	167	365	67	6	290	351
CO_2 output (μl.)	471	1854	1898	16	3109	1774
R.Q.	2·8	5·1	28·6	2·7	10·7	5·1

Acetate alone appeared not to be metabolized and malate oxidation was not affected by the addition of acetate (Table 10). The addition of cofactors (adenosine triphosphate, 'liver coenzyme concentrate', magnesium, or methylene blue) did not stimulate acetate oxidation.

Formation of citric acid

Liquid nitrogen-treated cells were incubated with malate, pyruvate, acetate, malate plus pyruvate, and malate plus acetate. After 3 hr. samples were taken for citric acid determinations by the method of Natelson, Pincus & Lugovoy (1948). Table 11 shows that most citric acid was formed from a mixture of malate and pyruvate, though the amounts formed from each of these substrates separately were greater than that formed from them both together. The presence of citric acid was confirmed chromatographically, using indicator and the Furth-Hermann reagent as developing sprays.

No citrate was formed from acetate.

Table 11. *Formation of citric acid*

Each boiling tube contained in a total volume of 5·0 ml.: 7·56 mg. cell N, 0·05 M-phosphate buffer (pH 6·5), 'liver coenzyme concentrate' 4 mg., ATP 4 mg., methylene blue 0·2 mg., 0·01 M-$MgSO_4$, and 0·04 M-substrate. Gas phase air; temp. 25°.

Substrate	Citric acid formed (μmole)
None (endogenous)	1·05
L-Malate	3·75
Pyruvate	3·10
Acetate	0·85
L-Malate + pyruvate	5·25
L-Malate + acetate	2·40

DISCUSSION

In common with much of the work done on the respiration of organic acids by micro-organisms, the picture presented by whole and cold-treated cells of *Zygorrhynchus moelleri* shows various differences. Owing perhaps to permeability barriers, intact cells of this organism are unable to oxidize the intermediates of the citric acid cycle at pH values near neutrality, whilst exhibiting great oxidative capacities for glucose and acetate. By reducing the pH to 3·4, all the cycle intermediates tested can be oxidized. Inhibitor studies with malonate have shown that at pH 6·8 diethylmalonate in fairly high concentration inhibits the oxidation of both glucose and acetate, though at pH 3·4 succinic acid oxidation is only slightly inhibited by malonic acid (in spite of the latter probably being able to enter the cells), and the inhibition becomes weaker as the experiment proceeds. Gray (1952*a*, *b*) has observed the adaptive formation of a malonic decarboxylase in *Pseudomonas aeruginosa* which relieved inhibition of succinate oxidation by malonate in that organism.

With cells frozen in liquid nitrogen it has been possible to demonstrate chemically many of the reactions associated with the tricarboxylic acid cycle. These are: the conversion of *cis*-aconitate to citrate, citrate to α-ketoglutarate, *cis*-aconitate to α-ketoglutarate, α-ketoglutarate to succinic semialdehyde, α-ketoglutarate to succinate (in the presence of malonate), succinate to a mixture of fumarate and malate, fumarate to malate, malate to oxalacetate (in the presence of cyanide) plus pyruvate, malate to citrate, pyruvate to acetaldehyde, and pyruvate to citrate. Whole cells are able to oxidize acetate very rapidly, but this property is lost when they are frozen, and no reaction products were found from acetate metabolism.

Malonate has been shown to inhibit the succinoxidase system competitively in cells treated with liquid nitrogen. The oxidation of α-ketoglutarate is also inhibited by malonate, but it was not established whether this was due to the inhibition of the α-ketoglutaric oxidase or to inhibition of oxidation of the succinate formed.

Sanadi & Littlefield (1952) and Kaufman, Gilvarg, Cori & Ochoa (1953) found that α-ketoglutarate oxidation requires coenzyme A and diphosphopyridinenucleotide and proceeds via succinyl-coenzyme A; in the absence of an oxidant α-ketoglutarate is decarboxylated slowly to succinic semialdehyde, the latter not being oxidized further by α-ketoglutaric oxidase (Sanadi, Littlefield & Bock, 1952). In view of these findings it is interesting to note that in the absence of coenzyme additions α-ketoglutarate is decarboxylated by *Zygorrhynchus moelleri* with the formation of succinic semialdehyde, but is not oxidized; when cofactors are added oxygen is absorbed and some succinate is formed (Table 8). A similar state of affairs might hold for pyruvate oxidation and acetaldehyde formation (Korkes, del Campillo, Gunsalus & Ochoa, 1951; Korkes, del Campillo & Ochoa, 1952; Littlefield & Sanadi, 1952).

Table 12. *Comparison of oxidative capacities of intact and liquid nitrogen-treated cells*

Comparison of q_{O_2}(N) of intact cells (from Table 1) and q_{O_2}(N) of liquid nitrogen-treated cells (from various tables). Endogenous respiration deducted in each case.

Substrate	Intact cells	Liquid nitrogen-treated cells	Intact cell activity remaining in treated cells (%)
cis-Aconitate	38·0	10·1	26·6
Citrate	197·2	2·0	1·0
L-Malate	295·6	15·8	5·3
α-Ketoglutarate	447·2	3·6	0·8
Pyruvate	530·6	2·9	0·5
Succinate	655·6	23·9	3·6

The oxidative abilities of cells treated with liquid nitrogen are very much less than those of whole cells (Table 12). The high rates of oxidation of some substrates by whole cells, particularly of succinate and acetate (which are oxidized more rapidly than glucose), tend to indicate that these substances are likely to play some part in the oxidative metabolism of glucose by this organism. Krebs (1943), in a similar connexion, remarks: 'Striated muscle is a tissue in which carbohydrate is the predominant substrate for oxidations. . .

It follows...that a substance which is *rapidly* metabolized in muscle may be expected to be associated with carbohydrate metabolism'.

With frozen cells the position is much less clear. The fraction of the oxidative capacity of the whole cells which remains after treatment with liquid nitrogen hardly exceeds 5%, except in the case of *cis*-aconitate (Table 12), and even here the relatively high percentage of the metabolic activity remaining in treated cells is probably due in part to the low activity shown by whole cells, which in turn may be associated with permeability barriers operative even at low pH values. 'Sparking reactions', such as those found with lupine mitochondria (Brummond & Burris, 1953) and *Azotobacter agile* extracts (Repaske & Wilson, 1953), in which the oxidations of pyruvate and acetate are catalysed by the presence of malate, are not shown either by whole cells or by cells treated with liquid nitrogen (Table 10).

Thus, although work with frozen cells has demonstrated that tricarboxylic acid cycle reactions can be carried out by this organism, any conclusions regarding their quantitative importance must remain tentative.

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Studies on a Soap Tolerant Organism: a New Variety of *Bacterium anitratum*

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SUMMARY: A soap-tolerant organism, commonly found in face-flannels and sponges, was compared with *Bacterium anitratum*, *Moraxella lwoffi* var. *brevis* and *Alcaligenes viscosus*. These organisms have many characters in common. It is proposed that *Bacterium anitratum* and related organisms should be included in the family Achromobacteriaceae and that the soap-tolerant organism be considered a new variety of *B. anitratum* with the provisional name *Achromobacter anitratum* var. *saponiphilum*. Some problems of classification of these and similar organisms are discussed.

The soap-tolerant organism had two major antigens; one was associated with the capsule and largely strain specific, the other was common to all strains and was present also in non-capsulated variants. Both antigens were detached from the cell on heating, the specific antigen partially and the common antigen almost completely; they could be demonstrated in the saline medium after suspensions had been heated at 100° for 60 min. and though detached from the cell, could still stimulate antibody formation. There was an antigenic relationship between the soap tolerant organisms and *Bacterium anitratum*.

Certain Gram-negative cocco bacilli isolated from a variety of pathological sources were named *Bacterium anitratum* by Schaub & Hauber (1948). Similar organisms have been variously named *Herellea vaginicola* (tribe Mimeae) (de Bord, 1939, 1942), B5W (Stuart, Formal & McGann, 1949) and *Moraxella lwoffi* var. *glucidolytica* (Piéchaud, Piéchaud & Second, 1951).

Gram-negative diplococci sometimes found in cerebrospinal fluid, distinguishable from the meningococcus by their ready growth on unenriched media at room temperature, have been named *Diplococcus mucosus* (von Lingelsheim, 1906, 1908; Cowan, 1938; McFarlan, 1941; Bray & Cruickshank, 1943). The majority of organisms from cerebrospinal fluid and other sources identified as *D. mucosus* are however identical with *Bacterium anitratum* (Seeliger, 1953) and differ from Cowan's strains (the first to be fully described) in their inability to reduce nitrates to nitrites or to liquefy gelatin, in the development of bacillary forms, and in feeble virulence for mice. Two other organisms, *Moraxella lwoffi* (Audureau, 1940) and *Mima polymorpha* (tribe Mimeae) (de Bord, 1939, 1942) appear to differ from *Bacterium anitratum* only in their inability to produce acid in peptone water sugar media.

The present work arose from the isolation of a soap-tolerant (ST) organism from face-flannels and sponges. It closely resembled *Bacterium anitratum* and preliminary studies showed that it also had many characters in common with saprophytic Gram-negative bacilli found in food and water, including *Alcaligenes viscosus*. As there have been widely differing opinions regarding the systematic position of *Bacterium anitratum* and similar organisms, the ST organism was studied in detail, including its antigenic structure, and its characters compared with strains of *B. anitratum*, *Alcaligenes viscosus* and some saprophytic organisms with similar properties; a single strain of *Moraxella lwoffi* var. *brevis* was also included. It was hoped that this study would clarify the taxonomy of what is at present an ill-defined group.

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GENERAL CHARACTERS AND TAXONOMY

Sources and isolation of strains

Soap-tolerant organisms were isolated on Lemco peptone agar containing 10% (w/v) of 'Ivory' toilet soap. One ml. inoculum was spread over the surface of the medium in a Petri dish and incubated at 25° for 7 days. ST colonies appeared earlier (2–3 days) than other soap tolerant organisms. The materials examined included residual moisture squeezed from 42 face-flannels and sponges, 30 specimens of infected urine, 14 faecal and 9 soil extracts (supernatant fluid from thick suspensions); the numbers of strains isolated were, respectively, 26, 1, 3 and 0. An additional strain was from an abscess where the predominant organism was a staphylococcus.

Bacterium anitratum: all twenty-seven strains had originally been isolated from pathological sources including cerebrospinal fluid, urine and bed-sores and included the 10 type strains of Ferguson & Roberts (1950), and NCTC 8101.

Alcaligenes viscosus group: three typical strains were obtained, all from milk. Six similar but non-viscid organisms were isolated from water, meat and milk. A further 3 strains included in this group liquefied gelatin.

Moraxella lwoffi var. *brevis*: a single strain was obtained from the National Collection of Type Cultures, NCTC 7976.

Cultural and biochemical methods

Morphological and cultural characters were determined from cultures on Lemco peptone medium. Incubation temperature was 25° or 37° according to the preference of the strain or 25° if there was no preference.

The substrate for fermentation tests was sterilized by Seitz-filtration and 1% (w/v) added to peptone water with Andrade's indicator; tests were incubated for 14 days. A 10% lactose agar medium, recommended by Chilton & Fulton (1946) for detecting late lactose fermenters, was used in addition to the usual 1% lactose peptone water.

Lipase was detected on an olive oil medium with neutral red as indicator. Horse-blood agar was used for haemolysis. Methods in general use were used in all other tests and uninoculated media, and known positive organisms were always included.

RESULTS

The morphological, cultural and biochemical data for all strains are summarized in Table 1.

Table 1. *Summary of morphological, cultural and biochemical characters of the soap-tolerant organism* Bacterium anitratum *and similar bacteria*

Characters common to all strains

Gram-negative cocci or bacilli; often pleomorphic; strictly aerobic; grow well on unenriched media and on MacConkey's agar at 25°; do not produce acid from 1 % lactose, sucrose, maltose, mannitol, glycerol; catalase-positive; indole-, H_2S- and VP-negative; anaerogenic; do not reduce nitrates to nitrites.

Characters not common to all strains

	ST	*B. anitratum*	*A. viscosus* *group*	*M. lwoffi* var. *brevis*
No. of strains	31	27	12	1
Capsules	+	+	− (5)	−
Motility	−	−	−	−
Growth at 37°	+	+	+ (5)	+
Growth at 44°	— (10 Tr)	+ (1)	−	−
Citrate utilization	+	+ (2)	− (3)	−
Acid from glucose	+ (1)	+ (1)	−	−
Acid from galactose	+ (1)	+ (1)	−	−
Acid from xylose	+ (1)	+ (1)	−	−
Acid from arabinose	+ (1)	+ (1)	−	−
Acid from 10 % lactose agar	+ (1)	+ (1)	−	−
Oxidase	−	−	−	−
Lipase	+	+ (1)	+	+
Lecithinase	+	+ (1)	+ (6 Tr)	Tr
Gelatinase	+	−	− (3)	−
Haemolysis	+	+	Tr	Tr
Soap tolerance	10 %	< 0·6–2·5 %	< 0·6 %	< 0·6 %
Litmus milk	CAD (1)	CAD (1)	Alk (6)	Alk

Tr = trace, C = coagulation, A = acid, D = digestion, Alk = alkaline. Figures in parentheses indicate number of exceptions to the general rule.

Special features of ST strains

The capsules are shown in Pl. 1, fig. 1. At 37° some strains were diplococcal or ovoid (Pl. 1, figs. 2, 3) but others grew as rods, filaments and bizarre forms which were also capsulated (Pl. 1, figs. 4–6). The rods frequently showed bipolar staining. Strains which were markedly pleomorphic at 37° were predominantly ovoid at 25°.

In Lemco broth there was good growth at 5, 25 and 37° but never more than a trace at 44°. Ten strains preferred 25 to 37°, the remainder were indifferent, or preferred 37°. In broth there was uniform turbidity, an iridescent surface ring and a ropy deposit.

Colonies on agar, after 1–2 days of incubation, were 1–2 mm. in diameter, circular with an entire edge, convex, smooth, glistening, greyish white, moderately opaque and markedly iridescent. Ten strains were viscid, the remainder butyrous in consistency. (All viscid strains grew better at 37° than at 25° and showed little pleomorphism.) On further incubation colonies increased in size and usually became radially striated or segmented, the segments being more translucent and less iridescent. After 5–7 days some strains showed surface papillae.

Non-capsulated variants were obtained by plating old cultures or subculturing from segments. The variant colonies were smaller, more translucent and less iridescent, butyrous or cheesy in consistency but never viscid. Some were spontaneously agglutinated in saline and serological tests suggested that surface antigens had been lost.

From two strains another type of variant was isolated. The colony was normal in form but in consistency was much more viscid; it never became striated or segmented. Morphologically the organisms resembled the normal form and were capsulated. Serological tests showed that there was no loss of surface antigen, and it appeared that this was a stable M (mucoid) form in comparison with the normal form which invariably gave rise to segments consisting of non-capsulated variants.

Gelatin was liquefied in 2–3 days, except for the non-fermenting strain which took 7 days. This strain produced an alkaline reaction in litmus milk, but the remainder coagulated milk by a rennin-like enzyme, followed by slow formation of acid and slight digestion. Changes occurred more rapidly if there was a residual cream layer in the medium. Using Hugh & Leifson's method, it was shown that acid production from glucose was due to oxidation, not to true fermentation. Haemolysis was slight. The MR test was variable. On Christensen's (1946) urea medium a few strains were weakly positive.

All strains grew well on Lemco agar containing up to 10% of toilet soap; they were equally tolerant of disinfectant soaps such as 'Lifebuoy' and 'Hexaphene'. The maximum tolerances of other organisms were: *Escherichia coli*, 2·5%; *Aerobacter aerogenes*, 2·5%; *Staphylococcus albus*, 0·6%, *S. aureus*, <0·6%; *Bacillus subtilis*, <0·6%.

The ST organism utilized soaps as the sole source of carbon when these were substituted for citrate in Simmons's (1926) medium; sodium oleate gave a more profuse growth than sodium palmitate or sodium stearate.

Nine strains that were tested failed to kill mice when injected intraperitoneally except in massive doses containing about 10^9 viable organisms. Similar numbers did not cause necrosis when injected intradermally into guinea-pigs.

Comparison of the ST organism with similar bacteria

Bacterium anitratum. This was an ovoid bipolar stained organism with less pleomorphism. Its colonies on agar were larger and more opaque and there was denser turbidity in broth. It grew better at 37° than at 25° and, except for one strain, grew well at 44°. It differed, too, in having a low soap tolerance and in not liquefying gelatin (Table 1). It formed a biochemically homogeneous group except that one strain did not produce acid from glucose, two did not utilize citrate, and one was lipase and lecithinase negative. Five strains had a viscid growth.

Alcaligenes viscosus group. The twelve strains examined were diplococcal or ovoid diplobacilli, some showed small capsules. Like some ST strains those able to grow were often highly pleomorphic at 37°. Abd-el-Malek & Gibson (1952) have remarked on the coccal morphology of some of their strains of *A. viscosus*. Three strains liquefied gelatin and differed from the non-fermenting ST strain only in their low soap tolerance (0·6 %). One was unable to grow at 37°. The typical *A. viscosus* strains differed from the non-fermenting *Bacterium anitratum* strain only in their viscous growth and in failing to grow at 44°. Also capsules were not demonstrated.

Moraxella lwoffi var. *brevis*. This organism, a diplobacillus, showed no pleomorphism at 37°. It differed from the non-fermenting strain of *Bacterium anitratum* in being non-capsulated and in failing to utilize citrate and to grow at 44°.

SEROLOGY

Materials and Methods

Suspensions were made in saline from 20 hr. agar cultures at 37° washed once and resuspended in saline, containing 1/10,000 thiomersalate. For agglutination they were equal in density to Brown's tube no. 3; 5 times as dense for immunizing rabbits and 25 times as dense for agglutinin absorption and for preparing extracts.

Preparation of antisera. Rabbits were immunized intravenously by five injections from 0·1 to 2·0 ml. of living organism at 5-day intervals and the serum collected 7–10 days later. The sera from two rabbits were pooled and preserved with 1/10,000 thiomersalate.

Sera were prepared against four ST strains (ST 1, 2, 3, 5) and two *Bacterium anitratum* strains, B 1 (NCTC 8101) and BT 1 (capsular type I, *B. anitratum*, Ferguson & Roberts, 1950).

Agglutination tests were incubated at 37° for 2 hr., and read after standing overnight at 5°.

Heated suspensions were kept at 100° for 1 hr., washed twice in saline and resuspended in saline. This washing was important to remove antigen detached during heating.

Extracts for precipitin tests were the supernatants from saline suspensions which had been heated at 100° for 1 hr. and centrifuged.

Precipitation tests. Twofold dilutions of extracts were mixed with an equal volume of 1/5 antiserum, incubated at 37° for 2 hr. and read after standing overnight at 5°.

Antigenic components of ST strains

All 31 ST strains were agglutinated to a high titre by the 4 ST antisera. Agglutination was rapid with formation of large floccules, different in size for each strain.

Absorption of each of the 4 ST sera with another strain or a non-capsulated variant (VST1) removed the common antibody but left a specific antibody which agglutinated its own strain to a low titre. The specific antigen appeared to be capsular as it was not demonstrated in non-capsulated variants. Only four strains, including ST 2 and ST 5, possessed related specific antigens.

Suspensions heated to 100° were only agglutinated by their own antiserum and failed to absorb common antibody. In precipitation tests with extracts of heated suspensions, however, both antigens were readily demonstrated. The common antigen produced large grey floccular aggregates which formed in a few minutes whereas those of the specific antigen were smaller, white and formed more slowly. Sera prepared against extracts gave titres of up to 640 and 5120, respectively, of both specific and common agglutinin, showing that the extracted antigens were still active agglutinogens.

Table 2. *Cross-agglutination with four strains of the soap-tolerant (ST) organism and their respective antisera*

		Titre with							
		Unheated suspensions				Heated suspensions			
Anti-serum	Absorbed with	ST 1	ST 2	ST 3	ST 5	ST 1	ST 2	ST 3	ST 5
ST 1	Nil	**5,120**	2,560	5,120	5,120	**40**	0	0	0
ST 2	Nil	20,480	**40,960**	20,480	40,960	0	**640**	0	40
ST 3	Nil	20,480	40,960	**20,480**	40,960	0	0	**160**	0
ST 5	Nil	10,240	20,480	10,240	**20,480**	0	10	0	**640**
ST 1	VST 1*	**20**	0	0	0	**40**	0	0	0
ST 2	VST 1*	0	**160**	0	0	0	**640**	0	40
ST 3	VST 1*	0	0	**0**†	0	0	0	**160**	0
ST 5	VST 1*	0	0	0	**20**	0	10	0	**640**

* Non-capsulated variant of ST 1.
† When tested against a suspension grown at 25° titre = 40.
Bold figures show homologous systems.

Sera prepared against heated suspensions also contained specific agglutinins with titres of up to 640. In contrast, common agglutinin was almost absent, a small amount (titres of 10 and 160) being found in only two of the four sera. This was in keeping with the other evidence that heating removed most of the common antigen from the cell. Two of the non-capsulated variants, VST 1 and VST 3, became autoagglutinable when the common antigen was removed by heating. VST 2 and VST 5, on the other hand, were autoagglutinable before heating and common antigen was hardly detectable in extracts. This suggested that variation involved the progressive loss of specific and common antigen and that loss of both antigens led to saline instability.

Effect of growth temperature on antigen production

Reference has been made to ten strains which grew better at 25° than at 37°. These strains were not agglutinated by the four antisera when grown at 37° and common antigen was barely detectable in extracts. It appeared that the formation of common antigen had been suppressed at 37°. In one such strain against which an antiserum had been prepared, specific antigen was readily demonstrated by agglutination and precipitation tests on cultures grown at both temperatures.

One strain, ST 3, when grown at 37°, was not agglutinated by a specific (absorbed) serum, whereas at 25° it was (Table 2). This was not due to the non-production of specific antigen at 37° as heated suspensions were agglutinated and specific antigen was present in extracts. In this case the common antigen appeared in some way to prevent specific agglutination.

Antigenic difference of a non-fermenting ST strain

The non-fermenting ST strain (Table 1) differed antigenically from the other strains. A suspension grown at 25° was agglutinated by the 4 ST antisera but only to 10–20% of titre. The strain did not absorb an appreciable amount of common antibody, but it appeared to have a common antigen related to but not identical with that of the other strains. Heated suspensions were inagglutinable; the specific antigen was not investigated.

A suspension grown at 37° was not agglutinated by any of the four sera, suggesting that the common antigen was not produced at this temperature.

DISCUSSION

A study of the morphology, cultural characters and biochemical reactions of the ST organism shows that it is closely related to *Bacterium anitratum*. Both organisms are similar to *Alcaligenes viscosus* and other saprophytic organisms found in food and water, which supports the suggestion that *Bacterium anitratum* and related organisms should be included in the genus *Achromobacter* (Brisou & Morichau-Beauchant, 1952; Brisou, 1953). It is proposed that the ST organism should be considered as a new variety of *Bacterium anitratum*, and, as the generic name *Bacterium* is now invalid (Judicial Commission, 1953), that both varieties should be included in the genus *Achromobacter* (family Achromobacteriaceae), the ST organism being named *A. anitratum* var. *saponiphilum*. By Rule 7 of the International Bacteriological Code of Nomenclature (1948), typical strains of *Bacterium anitratum* are automatically renamed *Achromobacter anitratum* var. *anitratum*.

The status of acid production in peptone water sugar media as a differential character for strains of these organisms is doubtful. Single strains of both *Bacterium anitratum* and the ST organism failed to ferment any sugars. In the latter case, the non-fermenting strain was agglutinated by antisera to the fermenting strains. Piéchaud *et al.* (1951) also observed serological cross-reactions between their fermenting and non-fermenting strains of *Moraxella lwoffi*, and Seeliger (1953) doubted whether fermenting and non-fermenting *Bacterium anitratum* strains should be considered as distinct species. Brisou (1953) has suggested that the genus *Alcaligenes* should be merged with the genus *Achromobacter*.

The significance of growth temperature ranges and optima in the classification of this group is also doubtful. It is not unusual for strains of well-defined species, isolated from different sources, to vary in their temperature requirements and ST strains showed such variation to some degree, while some members of the *Alcaligenes viscosus* group differed from one another only in their ability to grow at 37°.

There is no evidence that ST organisms are pathogenic, though they were found twice in pathological material. Viscous strains may be one of the causes of sliminess of face-flannels and sponges.

Two main antigens of ST organisms have been clearly defined. The 'specific' antigen is capsular and is not found in non-capsulated variants. It resists heating at 100° for 1 hr. and is partly detached by this treatment. It is largely strain specific and pure specific agglutinin can be prepared by absorption.

The common antigen is widely distributed, resists heating at 100° for 1 hr. and is completely (or almost completely) detached in the process. It is present in non-capsulated variants, but appears to be a surface antigen. In its general properties it resembles the Vi rather than the O antigens as the former are surface antigens, which are detached by heat and are heat-resistant (Stuart & Kennedy, 1948; Felix, 1952). With one strain there was evidence to suggest that it prevented specific agglutination of cultures grown at 37°.

On the basis of capsular antigens Ferguson & Roberts (1950) found ten serological types of *Bacterium anitratum* and observed cross-reactions with unabsorbed sera due to another antigen. Preliminary tests made in this study suggest that it may be similar to the common antigen of the ST strains.

Cross-reactions were observed between ST strains and four *Bacterium anitratum* strains. Antiserum to Ferguson & Roberts type I strain agglutinated all ST strains, and this appeared to involve the common antigen, but none was agglutinated by antiserum against NCTC 8101. None of the other organisms included in this study was agglutinated by ST or *B. anitratum* antisera.

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EXPLANATION OF PLATE

Films of ST strains from 18 hr. cultures on Lemco agar, incubated at 37°. Magnification, ×1250. Figs. 2–6 were stained with dilute carbol fuchsin.

Fig. 1. ST 1 stained for capsules by Howie and Kirkpatrick's method showing capsules.
Fig. 2. ST 1. Coccal forms.
Fig. 3. ST 3. Ovoid forms.
Fig. 4. ST 3. Rod forms showing bipolar staining.
Fig. 5. ST 21. Branched bizarre forms.
Fig. 6. ST 22. Long filamentous forms.

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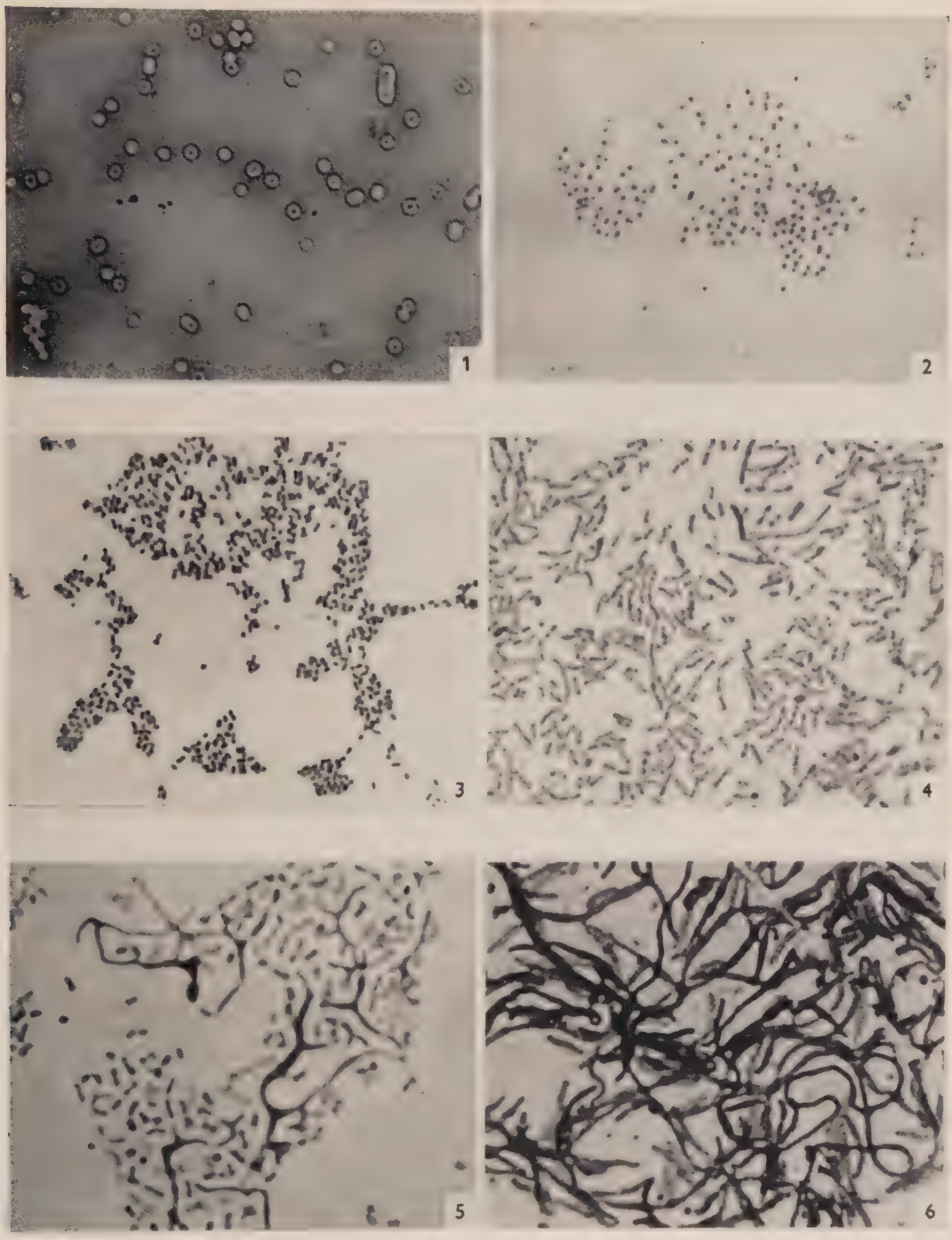

E. BILLING—NEW VARIETY OF *B. ANITRATUM*. PLATE 1

(*Facing p.* 260)

Erikson, D. & Porteous, J. W. (1955). *J. gen. Microbiol.* **13**, 261–272

Commensalism in Pathogenic Anaerobic Actinomyces Cultures

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SUMMARY: Conversion of a 'rough' typical strain of *Actinomyces israelii* of human origin to a 'smooth' soft form more tolerant of oxygen was found to result from the physical trapping within the mycelium of a few alien and facultatively anaerobic cells. Such cells, which commonly proved to be staphylococci, were isolated in single cell culture by micromanipulative techniques and then grown aerobically. Their presence affected the morphology and oxygen tension requirements of the host. In successfully balanced mixtures the colonies appeared homogeneous anaerobically and failed to grow aerobically. The commensal organism could most readily be detected in a water/gel system in stoppered bottle cultures. Similar associations were obtained from a variety of morbid material, especially that of bovine origin. It is suggested that the descriptions given by various workers at different times of a smooth soft micro-aerophilic organism little given to branching may cover: (1) *A. israelii* and associates; or (2) a distinct organism earlier described under the name of *A. bovis*.

Throughout the extensive literature on the anaerobic pathogenic actinomycetes, there are certain recurrent themes which have elicited much discussion. They are:

(1) In the animal host, even in closed lesions, all actinomycetes are accompanied by other micro-organisms (Klinger, 1912; Colebrook, 1920; Bates, 1933; Holm, 1950).

(2) Although on primary isolation strictly anaerobic, organisms may on subsequent cultivation tolerate some degree of oxygen tension (Bisset & Moore, 1949; Ludwig & Sullivan, 1952; Prévot, 1953).

(3) While the classic *Actinomyces israelii* produces a 'rough' irregularly shaped colony on the surface of complex media such as blood agar, together with a breadcrumb type of growth in liquid media, there may arise 'smooth' dissociants which are composed of shorter diphtheroid or bacillary cells less prone to branching and thus more evenly dispersed in liquid media (Lentze, 1938; Morris, 1951; Ludwig & Sullivan, 1952).

(4) Soft diphtheroid strains producing such 'smooth' colonies can also be obtained in primary isolation from morbid material of human origin (groups II and III of Holm, 1948; Slack, Ludwig, Bird & Canby 1951), and of animal origin (Magnusson, 1928; Kimball & Frank, 1945; Thompson, 1950). These strains are less strictly anaerobic than the 'rough' ones, and sometimes require carbon dioxide.

One particular aspect of these themes is presented here.

METHODS

The media employed throughout were the stock medium (S) or the dilution media (A to F) of Erikson & Porteous (1953). Anaerobiosis was obtained by flushing the container vessels with nitrogen in the manner described in that paper. Water/gel cultures were prepared in small screw-capped bottles according to the method of Heald, Krogh, Mann, Appleby, Masson & Oxford (1953), with the modification that the concentration of agar in the solid layer was only 0·5 %. When this nutrient layer incorporating the inoculum had set, the bottle was carefully filled with freshly-boiled cold sterile water up to the rim and the cap screwed down.

Micromanipulations were carried out according to the methods of Erikson & Masson (1954). Daily examinations of growing cells were made by means of phase-contrast and bright-field microscopy.

Seven human strains of typical *Actinomyces israelii* were used; all had been recently isolated from cases of actinomycosis, and five of them had already been shown to have similar powers of utilizing glucose with the production of lactic acid (Erikson & Porteous, 1953). In addition, about 120 specimens of morbid material from animal sources, reputedly actinomycotic in origin, were obtained from the local slaughter-house and from veterinarians in different parts of the country. The specimens were examined histologically, and attempts to obtain cultures were made in almost all cases, using a variety of customary procedures. The great majority of the specimens were bovine in origin, but a few were from sow udders.

RESULTS

It will be more convenient to consider first the practical details involved in the above aspects (2) and (3) of the problem: conversion of hard 'rough' to soft 'smooth' growth with increased tolerance of oxygen and turbidity of short-celled liquid cultures.

Spontaneous origin of 'smooth' growth

After about 18 months of continuous weekly subculture of seven human strains of *Actinomyces israelii* in S (glucose heart-broth casein digest) liquid medium, during which period the yields and appearances of the organisms were much the same for all strains, it was noticed that one particular strain (1394) was growing more profusely than the others. When the routine microscopical examinations made before subcultivation were checked, it was found that the micro-colonies of strain 1394 were on the whole characteristic if rather shrunken (Pl. 1, fig. 1), but that their component cells were shorter and less regularly branched (Pl. 1, fig. 2). When plated out, the organism seemed homogeneous, even though the cultures were rather smoother than usual. Liquid cultures still retained their clarity, but after four more subcultures a certain 'incohesiveness' had become quite perceptible, i.e. normally discrete compact colonies when gently shaken in the tube fragmented readily, thus bringing about a slight turbidity of the medium.

At this stage strain 1394 was inoculated into tubes of the various dilution media A to F (six replicates of each); their subsequent history was as follows:

1st week: S tubes all turbid; A to C similar; D to F typical growth and clear media:

2nd week: from D medium to D medium—short to moderately long filaments, sometimes branched, and short rods (Pl. 1, figs. 3–5); from F medium to F medium—rods and short filaments cohering with small cocci (Pl. 1, fig. 6–9).

3rd week: to D medium from F medium—filaments at margin of microcolony, centre less defined (Pl. 1, fig. 10); to F medium from F medium—similar; plate of D medium from D liquid—smooth glistening colonies showing short rod and diphtheroid components (Pl. 1, fig. 12).

4th week: to D medium from D medium—much fragmentation, some longer filaments; to S medium from D medium—similar, fewer filaments.

During this period of 4 weeks the original profuse growth had also been subcultured aerobically in S medium with remarkable degree of continuity for 6 subcultures, but now in the 8th subculture it showed a decided falling off. Single smooth colonies from an anaerobic plate of medium D were then suspended so that the cells of any one colony were used as inoculum for both aerobic and anaerobic cultivation, with these results:

Aerobic: S medium mostly turbid—short rods and cocco-bacilli; A to D media—gradually more rods and short filaments; E and F media the clearest fluid and most typical branched filaments;

Anaerobic: S medium mostly clear—rods and cocci cohering with short filaments (Pl. 1, figs. 6, 7); A to D media mostly rods and filaments; E and F media more branching of filaments, all clear.

When these various cultures were subcultured four more times the aerobic cultures died out while the anaerobic ones presented much the same picture as that noted in the 4th week above. By repeated subculture of these growths in the more dilute media D to F, we succeeded by about the 12th week in stabilizing a form which appeared pure. Colonies in liquids were discrete and cohesive, even in the richest S medium, and, although the growth was a little soft to the touch, no clouding of the medium occurred at any stage. Colonies on slopes were raised and nodular, though smooth and glistening instead of rough and pebbled.

A return was also made to the slope and shake cultures which had been prepared as a routine before beginning these series of tests. The shake cultures appeared characteristic, but were no longer viable. The slope cultures exhibited a considerable amount of soft spreading growth, with isolated typical but rather smooth colonies at the margin. Morphologically they were similar to the cell complexes described above as appearing during the first week in S liquid media. It was therefore considered possible that: (*a*) the organism had been superseded by a contaminant; (*b*) the organism had undergone a spontaneous dissociation, and the two types had co-existed for a time until the smooth dissociant had supplanted the original form; or, (*c*) the organism was living commensally with a contaminant whose numbers varied according to cultural conditions and so affected the gross and particular appearances of the host.

The fact that normal aspect and morphology tended to be associated with the less rich dilution media D to F favoured hypothesis (*c*). Yet the contaminant, if any, did not seem to be separable by means of ordinary dilution and plating procedures with the media and anaerobic conditions used. Moreover, the aerobic liquid cultures mentioned earlier had died out, and it was therefore doubtful whether an aerobic or micro-aerophilic organism had been present from the first appearance of the phenomenon. In the circumstances it was decided to attempt single-cell isolations.

Single-cell isolations by micromanipulation

Four stocks of organisms of strain 1394 were used to provide the initial pools for micromanipulation (Erikson & Masson, 1954). The immediate history of these four stocks was as follows:

Stock (1): 5-day anaerobic growth in liquid medium A from single colony on a slope of F medium.

Stock (2): 5-day anaerobic growth in liquid medium A from single colony on a D medium plate.

Stock (3): 5-day anaerobic growth in liquid medium A from culture in liquid medium D derived from a smooth colony on medium D plate.

Stock (4): 5-day anaerobic growth in liquid medium A from liquid medium S derived from a culture in liquid medium F.

Stocks (1) and (2) were the more typical in appearance, while stocks (3) and (4) were softer and the component cells less coherent, but the medium was clear before shaking.

All four stocks were used in the original series of micromanipulative experiments which, as far as isolation of a typical actinomycete was concerned, represented the many failures mentioned in the earlier work of Erikson & Masson (1954). At that time efforts were confined to attempts to grow the cells within the droplets in the oil chamber, with flushing of the system repeatedly with nitrogen to decrease the amount of dissolved oxygen in the oil and medium, and subsequent incubation of the chamber in an anaerobic jar. The only cells which did develop under these conditions were certain short cell elements in stocks (1), (2) and (4). These short cell elements multiplied in the droplets as cocci, and when transferred to slopes of nutrient agar and incubated aerobically grew vigorously as a pure culture of Gram-positive cocci on repeated subculture. They were subsequently identified as *Staphylococcus albus* by comparison with an authentic culture. The droplets of medium that were uninoculated all proved sterile. Nor was any growth obtained in the series of droplets sown with stock (3), the single cells that had been separated in this instance being preponderantly rod- or filament-shaped.

As will be seen from Pl. 1, figs. 6–9, it was often difficult to decide whether or not the coccoid cells evident in such growths in stock cultures were in fact attached to the longer cells. When the charts giving the positions and shapes of every cell in the droplets were examined, it was apparent that an undue proportion of the short cell elements had been transferred to the slide. This was explained by the selective action of capillarity within the very slender tip of the finely drawn pipette with which the stock culture was sampled for the initial pool of cells. The preponderance in certain slides of these round cells was therefore not at first regarded with too much misgiving, but when it was found that only these cells grew in a system which de Fonbrune (1949) had described as unsuitable for the development of strict anaerobes, the evidence seemed conclusive that they were indeed alien cells.

Cultivation of single filaments or diphtheroid rods, with ensuing characteristic mycelial growth, was not achieved until the period within the oil chamber had been decreased to the minimum and the cells were incubated on solid media in an anaerobic slide-carrier drum (Pl. 1 of Erikson & Masson, 1954), or in semi-solid media in a water+gel system in stoppered bottles (Fig. 2 of Erikson & Masson, 1954). The mode of growth was similar in all instances, whether the initial cells were taken from the shorter diphtheroid forms or from slender filaments with the occasional club-shaped swellings that may be encountered particularly in liquid glucose-containing media (Pl. 2, fig. 15); or from the relatively longer and more homogeneous filaments developing between coverslip and agar in a slide culture (Pl. 2, figs. 16, 17). As recorded earlier (Erikson, 1940, pp. 16–18, 54–7, figs. 1–14), the growing cell elongates and in doing so may remain straight producing a branch, which itself elongates and branches in other directions (Pl. 2, figs. 20, 21); or it may bend at an angle, and immediately after branching a septum may be formed, allowing the new branch to slip sideways in characteristic angular apposition (Pl. 2, fig. 20).

The variety of planes through which these delicate filaments pass even in the restricted space between coverslip and agar is obvious from the fact that some portion of the minute growth is constantly out of focus (cf. Colebrook, 1920, who stated that the organism seldom ran straight for more than 20 μ.). Yet by focusing up and down the continuity of the cells could always be determined. Thus, in Pl. 2, fig. 18, the growing tip of the uppermost branch appears more bud-like and clavate than it really was, simply because the branch was beginning to turn over and the tip to burrow into the agar; and in Pl. 2, fig. 20, the apparent inequalities in diameter of the V-shaped growth at the bottom of the picture are due to the many slight twists in orientation, as well as to the development from one limb of a lateral branch which is almost entirely out of focus. Yet the slenderness of the uppermost portion of this filament, which after being cut off from the initial right-angled parent cell had slipped sideways, was real—not an artefact—and very characteristic of one mode of growth on solid media. In Pl. 2, fig. 21, the very beginning of a small branch can be seen in the centre right as a minute almost globular bud with very slender stalk. The close overlaying of different branches, which in fixed stained preparations can sometimes give the impression of enlarged or fused cells, is demonstrated in Pl. 2, fig. 19, where four distinct filaments have crossed in the centre (cf. Pénau, Hagemann, Velu & Peyré, 1954: Pl. 1, fig. 7, for similar artefacts in growth of *Streptomyces griseus*).

When the development of such minute mycelia produced by different strains was examined at intervals in a large number of slide cultures, it was found to follow the classical lines already described. In pure cultures derived from known cells no cocci or cells that could possibly be described as spores (cf. Morris, 1951) appeared at any stage. The colonies developed in characteristic fashion with a somewhat rough or pebbled surface, due to the constant interlacing of filaments branching in varying directions (Pl. 2, figs. 13, 14). Sometimes a few aerial filaments could be seen. Their emergence appeared dependent upon a variety of physical and chemical factors such as composition and consistency of medium, degree of humidity in culture vessel, and local surface tensions. In young colonies they were quickly overgrown, and in old ones they withered away; in no instances did they exhibit any reproductive function.

The success of the water+gel system (Erikson & Masson, 1954: fig. 2) for the cultivation of single cells of *Actinomyces israelii* led to the general use of a modified method based on that of Heald *et al.* (1953), which proved unexpectedly valuable both for the maintenance of stock and the detection of any impurities.

Use of water+gel system for detection of commensals

The use of shake cultures, particularly in rich glucose-containing media, has long been found satisfactory (see Wright, 1905) in the cultivation of anaerobic actinomycetes which, according to size and vigour of inoculum, tend to grow in a dense zone about half an inch below the surface when incubated aerobically. In the regular routine of weekly subculturing over a period of 2–3 years, it was constantly found that the colonies in such agar deeps (using S medium and 1–1·5% agar) retained their nodular circumscribed character. Only when a gross contamination was present was there any 'tailing' visible, reminiscent of various eubacterial growths in agar depths. In the case of the strain 1394 'soft' cultures, the single colonies towards the bottom of the tube or bottle showed no such spreading downwards into the agar, and consistently exhibited an entire outline though it was somewhat smoother than usual. No surface growth was ever apparent. When, however, the shake cultures were made in bottles layered with water in the manner described in the section on Methods, two noteworthy facts were evident: (1) the quantity of growth in the gel was considerably greater than in duplicate sets sown from the same suspension into the same medium, but incubated without a water layer—whether aerobically or anaerobically; (2) in certain bottles of strain 1394 a smooth soft growth composed mainly of cocci (Pl. 1, fig. 11) appeared at the medium/water interface.

These observations were repeatedly confirmed. In the case of the pure line strains, the good yield obtained by this method was further enhanced if, after

5–7 days, the supernatant water was removed and carefully replaced with fresh sterile water. Growth then continued for another 7–10 days, especially in a soft (0·5 %) agar, and seemed to follow from the removal of the acid products of metabolism which were found in the initial water phase. The provision of such a water layer, into which possibly toxic substances arising from the growth of the dominant organism were able to diffuse, also proved to be the readiest way of securing a favourable environment for the demonstration and characteristic growth of those few alien cells trapped within the actinomycete mycelium. Atypically smooth soft growth on nutrient agar slopes which yet showed clarity in anaerobic liquid cultures, and whose cells were so preponderantly diphtheroid in aspect (cf. Pl. 1, fig. 2) that it was difficult to detect (microscopically) shorter coccoid cell elements therein, often showed by this gel + water method a minute smooth colony of cocci at the gel + water interface. At the same time the water + gel controls remained sterile. The effect of adding alien cells to established pure line cultures was then examined.

Admixture of alien cells to pure Actinomyces israelii *strains*

Six morphologically different aerobic organisms, selected at random from common contaminants found in animal lesions (two staphylococci, one streptococcus, one diphtheroid, one sporing rod, one coliform organism) were added in minute quantities (the organisms which adhered to a needle-tip pressed lightly against a single colony) to each of six young replicate broth tubes of a pure culture of *Actinomyces israelii*. These cultures were then incubated anaerobically, continued in regular weekly subculture for 3–4 months, then later freeze-dried and opened 1 year later. The cultures with the coliform and diphtheroid admixtures grew very poorly and ultimately died out. A moderate growth of the actinomycete was obtained with the streptococcus and spore-former mixtures, but the fact that they were mixtures was evident after a few generations. The staphylococcal mixtures continued growth in the manner described for the soft culture of strain 1394: the alien cells found the conditions unfavourable for growth to any considerable extent as long as the cultures were maintained anaerobically and subcultured frequently, but were carried over in small numbers within the network of granules, ready to multiply whenever a more suitable substrate was supplied or when the cultures were left standing in an atmosphere of air. These cultures survived freeze-drying and produced a typical soft growth in agar shakes.

'Associates' in animal lesions

It is not possible to give data concerning primary associates of actinomycetes in the animal material examined, for closed lesions removed under aseptic conditions were not available. All the specimens received were considerably and sometimes very grossly contaminated, mainly with staphylococci but also with streptococci, diphtheroids, and a great variety of other organisms. This was particularly true of the bovine soft tissues such as cheek and retropharyngeal glands. Of about 120 specimens obtained from various veterinary sources only *c.* 15% showed on microscopical examination any branching filamentous or diphtheroid cells which could possibly be ascribed to actinomycete growth. 'Clubs' were found in not more than 5% of cases. Cultures which showed some characteristics of anaerobic actinomycetes were obtained in 10% of cases, as follows: 3 from bovine jaw; 2 from bovine lung; 4 from bovine soft head tissues; and 3 from sow udder.

Of the bovine specimens two, which were obtained locally, were outstanding cases of advanced actinomycosis. One specimen (J 2) was a jawbone with a large cavity which was filled with pus containing granules which crushed easily and showed a beautiful text-book picture of 'clubs'. Microscopically, Gram-positive branching filaments with some coccoid bodies dominated the smears. The culture obtained was somewhat soft in the first few generations, but was undoubtedly *Actinomyces israelii*; when subcultured during 2 years it showed no marked morphological or biochemical differences from human strains. The other specimen (L 1) was an entire lung oozing pus in which there were abundant egg-shaped granules. These granules when washed free from pus cells were smaller than those in specimen J 2, and the clubs were also smaller. The smears revealed filaments which were much shorter and less branched, with a greater proportion of coccoid elements. The organism that was isolated grew rather poorly in a soft short-filamented form, but with further purification it was also shown to be *A. israelii*. Neither of these organisms was as vigorous in artificial culture as were the human strains, and it is probable that each continued to harbour a few coccal cells which eventually swamped the cultures when they were unavoidably put aside for a time.

All the other bovine organisms were isolated from material which was far from fresh or had been sent through the post; these specimens carried such a mixed flora that it was impossible to feel any confidence concerning the etiological role of the isolates. In all instances they were the smooth soft type of organism, often growing with difficulty and yielding inconsistent results. Few of them survived any lengthy period of cultivation, and this was attributed to the impairment of vigour following on severe microbial competition in the tissues. Yet all showed more resemblance to *Actinomyces israelii* than to the pre-eminently diphtheroid *A. bovis* described earlier (Erikson, 1940).

During the latter part of this work some cultures isolated from bovine material by various veterinarians were received which showed very little, if any, branching of filaments and production of mycelium. When received on such complex media as blood agar, the very small soft smooth flat colonies sometimes showed an extremely pleomorphic picture of short rods, coccoid bodies, and occasional branching filaments of varying widths which resembled the schematic diagrams of Morris (1951). One such culture was dissected by means of the micro-manipulative methods of Erikson & Masson (1954), and an aerobic Gram-positive coccus was obtained from it in single-cell culture. The anaerobic diphtheroid element, which was also isolated in single-cell culture, grew well in a semi-solid yeast + peptone + lactate medium, suggesting that this organism might be related more closely to the propionibacteria (see Gutierrez, 1953).

All three specimens of sow udder contained orthodox granules surrounded by clubs, and relatively few contaminants. The organisms isolated were definitely strains of *Actinomyces israelii*. One of these strains was previously shown (Erikson & Porteous, 1953) to be culturally and biochemically identical with the human strains.

DISCUSSION

After summarizing the relevant literature in a discussion on this subject written 15 years ago, Erikson (1940) stated that 'the *israeli* type is preponderantly human in incidence, although it may occur elsewhere, notably in swine and occasionally in cattle; while the soft-growing form is most often found in the jaws—though again also in other regions—of cattle'. On the basis of our present evidence we can confirm the pre-eminence of *Actinomyces israelii* in actinomycosis of man and of pigs, but we must also admit that it can be found in the jaws and other regions of cattle exhibiting the gross pathology of the disease. Thompson (1950) found one case of *A. israelii* and fourteen soft *A. bovis* types in lumpy jaw of cattle.

The discovery that a typical strain of *Actinomyces israelii* could harbour a number of alien cells without showing obvious signs of contamination so long as the cultures were maintained under anaerobic conditions and subcultured regularly illumines aspects (2) and (3) of the problem outlined at the beginning of this paper, namely that of the conversion of hard 'rough' to soft 'smooth' growth. All the phenomena described for strain 1394—tolerance of oxygen, more profuse growth of short cell elements, turbidity in liquid media and soft smooth colonies on solids—are those which have been described in the literature as characteristic of various atypical non-israelii forms of *Actinomyces*. The marked inconsistency of the bovine forms which we obtained and their tendency to die out when transferred to simpler media are features indicative of contamination. They also closely parallel the experience of Magnusson (1928), who, referring to 54 isolates from bovine lumpy jaw, stated: 'it is well nigh impossible to test this organism...for it grows quite differently on different occasions, although the same culture strains and the same medium are employed'.

The possibility that associated bacteria influence the mode of growth and the morphology of the actinomycetes was suggested long ago by Alissow & Skworzow (1898). Our experiments involving deliberate contamination of pure cultures of *Actinomyces israelii* demonstrate the ability of vigorous young growths to incorporate certain random associates, at least for a period, without major disturbance in the economy of the dominant organism. The ease with which such intimate mixtures can be carried on to isolation plates can readily be granted if we bear in mind the low viability ratio of single cells of the actinomycete (Erikson, 1940, pp. 14–17; Erikson & Masson, 1954), the probability that many colonies are derived from small filamentous complexes, and the experience of Holm (1950) based upon the examination of 650 human specimens, who graphically described the associates as being 'found grouped between and up against the definitely characteristic, often delicately coiled, Gram-positive actinomyces filaments'. It is also significant that some of Holm's isolates are of the soft smooth type, and that all have been cultivated on very complex media.

Other workers, e.g. Beerens (1953) have classed human and bovine actinomycetes together with various corynebacteria. Since the cultures of Beerens developed a pinkish tinge in their soft smooth colonies, it seems likely that they all were indeed corynebacteria. In our experience no genuine actinomycetes with anaerobic requirements have at any time displayed any pigmentation. On the other hand, there does seem to be another type of microaerophilic diphtheroid organism to be found in bovine lesions. This is the organism described as *Actinomyces bovis* by Erikson (1940), which was isolated from soft tissues in Australian cattle. This organism differed serologically and biochemically from *A. israelii*, and produced very small flat colonies dissimilar in many respects from the soft smooth types of growth encountered during the present investigation. Certain strains resembling this *A. bovis* form were received towards the close of this work, as mentioned earlier, from various veterinarians in this country—in particular from Mr M. B. Hawksley of the Royal Veterinary College, London. His strains were serologically distinct from *A. israelii* (personal communication) and our preliminary examination revealed biochemical differences. The question of the generic status of *A. bovis*, however, is yet to be settled, namely whether the organism is indeed an *Actinomyces* sp. or is more properly to be considered a *Propionibacterium* sp., for branching was infrequent and dependent on cultural conditions.

Our experience leads us to believe that it is now time for a re-appraisal of the concept of smooth soft forms of anaerobic actinomycetes; a concept which may include different associations of *Actinomyces israelii* living commensally with other organisms or that of a quite distinct organism.

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EXPLANATION OF PLATES

(All phase contrast preparations.)

Plate 1

Fig. 1. Filamentous margin of microcolony of 'soft' growth of strain 1394 in liquid S medium, 10 days. (× 2160.)
Fig. 2. Portion of growth in Pl. 1, fig. 1, compressed between slide and coverslip, showing component cells mainly rod-like with little branching. (× 2160.)
Fig. 3. Short cell elements in D liquid culture of 1394, 6 days. (× 2160.)
Fig. 4. Occasional filamentous extension in same culture as in Pl. 1, fig. 3. (× 2160.)
Fig. 5. Rarer characteristic branched growth from same culture as in Pl. 1, fig. 3. (× 2160.)
Fig. 6. Cell complex in F liquid medium, showing irregular elements and some degree of lysis, 10 days. (× 2160.)
Fig. 7. Another replicate of same culture as in Pl. 1, fig. 6, showing vigorous growth of rods and short filaments, with a coccus simulating a bud. (× 2160.)
Fig. 8. Portion of growth from same tube as in Pl. 1, fig. 6, showing cocci adhering to short rods so that occasionally they appear stalked. (× 3360.)
Fig. 9. Another field in same specimen as in Pl. 1, fig. 8, showing clumping of cocci. (× 3360.)
Fig. 10. Another replicate of same culture as in Pl. 1, fig. 6, showing partial lysis of microcolony. (× 2400.)
Fig. 11. Strain 1394 at medium/water interface in water/gel S bottle culture, showing almost pure culture of cocci in smooth soft growth, 7 days. (× 2160.)
Fig. 12. Strain 1394 from a smooth colony on D agar medium incubated anaerobically, showing mainly diphtheroid and rod-shaped elements, 7 days. (× 2160.)

Plate 2

Fig. 13. Pure single-celled strain (Wills) plated on S medium, showing small colony with rough pebbled aspect and occasional aerial filament, 12 days. (× 1500.)
Fig. 14. Enlargement of colony in Pl. 2, fig. 13. (× 3000.)
Fig. 15. Same strain grown in liquid S medium, showing short segmented filaments and occasional swollen club-shaped cells, 10 days. (× 1500.)
Fig. 16. Progeny of culture in Pl. 2, fig. 15, in S slide culture, showing margin of microcolony with longer more homogeneous filaments developing between coverslip and medium, 5 days. (× 1500.)
Fig. 17. Enlargement of part of Pl. 2, fig. 16, showing twisting filaments at edge and dense bodies within one long basal filament. (× 3000.)
Fig. 18. Another field of same specimen as in Pl. 2, fig. 16, showing the growing tip of the uppermost branch burrowing into the agar and so presenting a clavate appearance. (× 3000.)
Fig. 19. Another field in same specimen as in Pl. 2, fig. 16, focused upwards to show an artefact—the appearance of an enlarged cell where four distinct filaments have crossed (× 3000.)
Fig. 20. Another field of specimen in Pl. 2, fig. 16, showing characteristic angular apposition resulting from the slipping sideways of V-shaped branch. (× 3000.)
Fig. 21. Another field of same specimen in Pl. 2, fig. 16, showing overlaying of different branches, and at centre right the very beginning of a small branch in the form of a minute globular bud with very slender stalk. (× 3000.)

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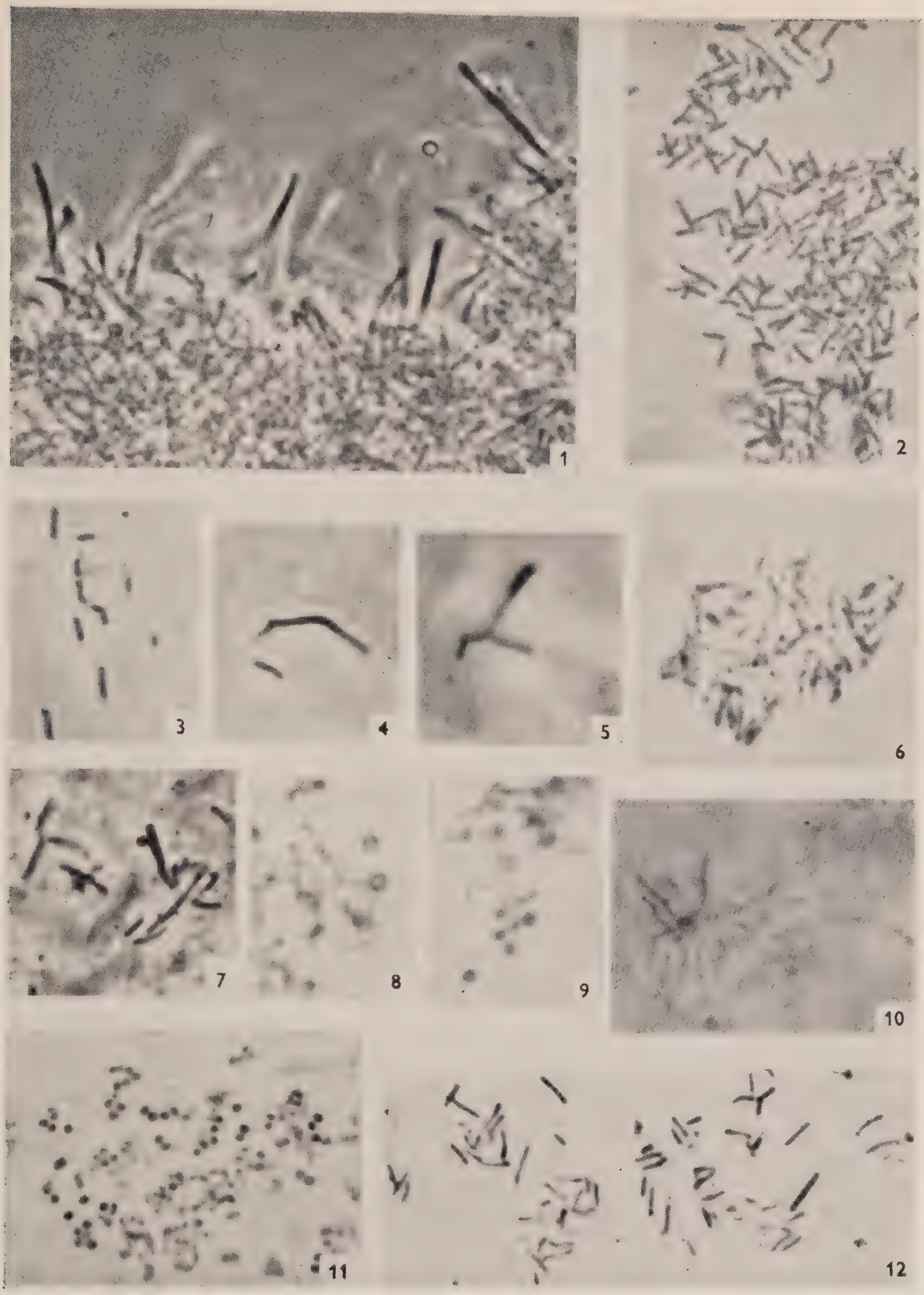
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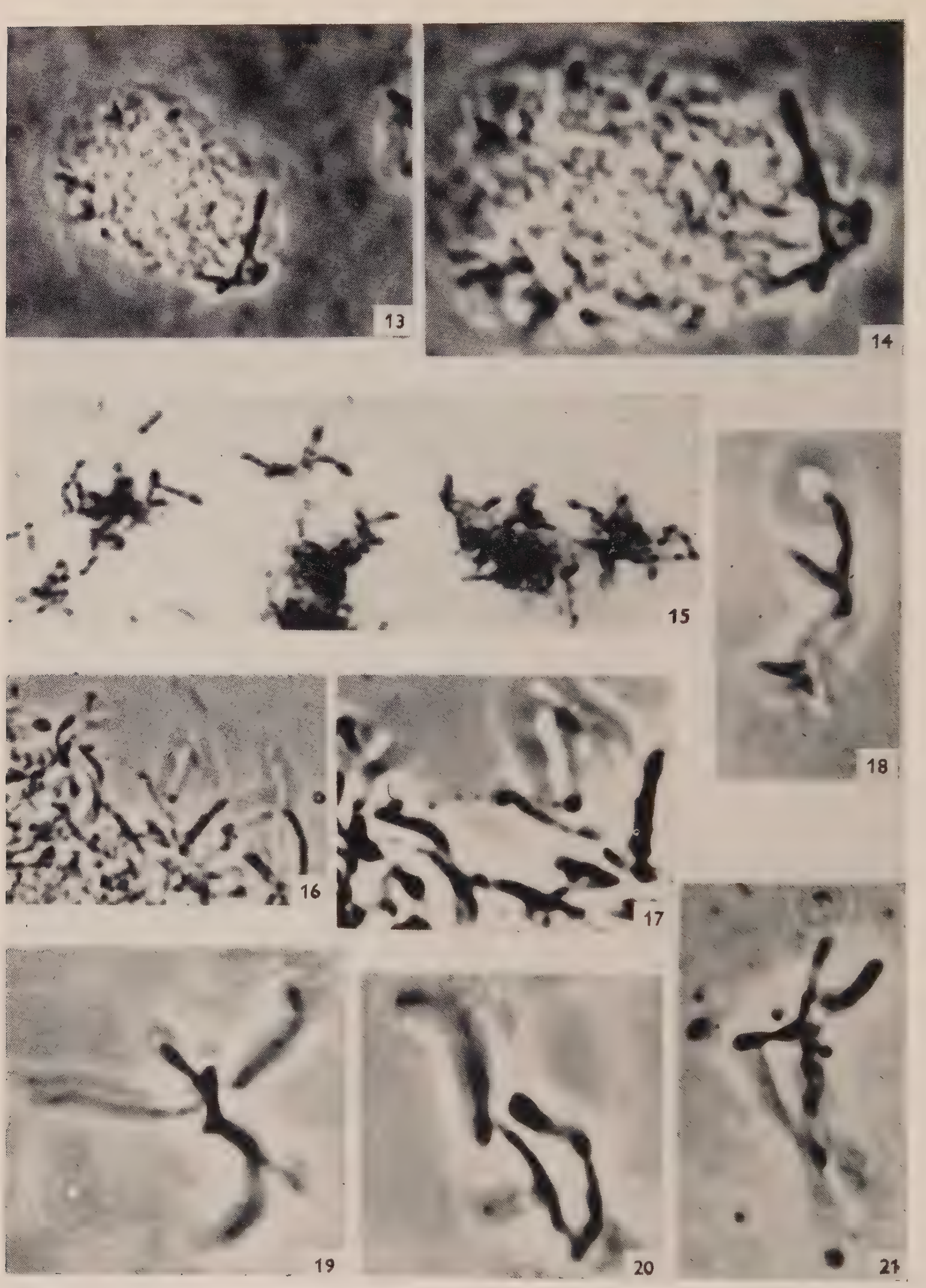
D. Erikson & J. W. Porteous—Commensalism in anaerobic actinomycetes.
Plate 1

(Facing p. 272)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D. Erikson & J. W. Porteous—Commensalism in anaerobic actinomycetes.
Plate 2

Citri, N. & Grossowicz, N. (1955). *J. gen. Microbiol.* **13**, 273–278

A Partially Defined Culture Medium for *Trypanosoma cruzi* and some other Haemoflagellates

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SUMMARY: A partially defined medium for cultivation of blood flagellates has been developed. In this medium blood was replaced by haematin, crystalline serum albumin and a series of simple growth factors. The medium supported growth of *Trypanosoma cruzi*, *Leishmania tropica*, and a few other strains of Trypanosomidae. The growth on this medium was comparable to that obtained on optimal blood-containing media; no change in growth was found after prolonged serial subcultivation.

The failure to propagate haemoflagellates in the absence of erythrocytes (Little & Oleson, 1951) or serum (Lwoff, 1951) has been a major obstacle in analysing the nutritional requirements and metabolic activities of this important group of parasites. A medium in which blood was replaced by tomato juice, bovine albumin and haematin, was described briefly by Citri & Grossowicz (1954*a*). The tomato juice has now been replaced by a number of known growth factors. Thus a medium is at hand which supports optimal growth of *Trypanosoma cruzi* and other haemoflagellates under partially defined conditions and which permits a better analysis of the growth requirements of these organisms.

METHODS

Organisms. All the organisms used in this work were kindly provided by Prof. S. Adler (Jerusalem). The strain of *Trypanosoma cruzi* now used (Culbertson strain) was obtained in 1949 from Dr T. S. Hauschka (Philadelphia) who kept it on Senekjie's (1943) medium. The strains had been maintained in the Department of Parasitology, Hebrew University, Jerusalem, on Adler's medium (Adler & Theodor, 1926) before the present work.

Stock diphasic medium. At the beginning of this work a satisfactory modification (Table 1) of the diphasic medium of Chang (1947) served as a source of cells for nutritional studies. It was made up as follows: solution (B) (20–25 ml.) was dispensed in 100 ml. Erlenmeyer flasks, autoclaved 15 lb/sq.in. pressure for 20 min. and allowed to harden in upright position to form a solid base. This base was overlaid with 15 ml. of solution (C).

Stock liquid medium. In the course of this work a simpler medium (TJ) was developed, in which a mixture of tomato juice, haematin and crystalline serum-albumin completely replaced blood. The composition of this medium was as follows: NaCl, 0·4 g.; $Na_2HPO_4.12H_2O$, 0·3 g.; KH_2PO_4, 0·05 g.; casein hydrolysate (enzymic, Bios Laboratories, Inc., New York), 1·0 g.; distilled water to 95 ml. This was autoclaved. To this autoclaved basal part of the medium were then added aseptically sterile haematin 1·0 mg. in 1 ml.; bovine albumin fraction V (Armour Laboratories, Chicago), 50 mg. in 1 ml.; tomato juice, 3·0 ml. The final volume was 100 ml.

Table 1. *Stock diphasic medium*

Solution A		Solution B	
Neopeptone (Difco)	20·0 g.	Solution (A)	600 ml.
Beef extract (Bovril)	6·0 g.	Agar-agar	9·0 ml.
Glucose	2·0 g.	Human haemoglobin solution*	30 ml.
NaCl	4·0 g.	Solution C (liquid overlay)	
KH_2PO_4	0·5 g.	Solution (A)	400 ml.
$Na_2HPO_4.12\ H_2O$	3·0 g.	ACD human plasma†	40 ml.
H_2O distilled	1000 ml.	Tomato juice‡	8 ml.
pH 7·8		Penicillin	24 mg.
		Streptomycin	40 mg.

* Prepared by laking 1 vol. of erythrocytes (human) in 3 vol. of distilled water; the ghost cells are removed by centrifugation.

† Both erythrocytes and plasma were obtained from citrated (ACD) blood batches discarded by the Blood Bank.

‡ The juice was squeezed from ripe tomatoes, strained through gauze, filtered through paper and then Seitz filtered.

The TJ medium served subsequently as a stock medium for *Trypanosoma cruzi* and *Leishmania tropica* and as a starting-point for the compounding of the partially defined medium to be described. The TJ medium and other media derived from it were dispensed in 5 ml. lots in 18 × 150 mm. test tubes, optically matched for turbidimetric measurements.

Growth determination. Cultures were incubated at 28° sloped at an angle of 15° from the horizontal. Growth was determined at intervals of 3 days by direct measurement of the optical density at wavelength 650 $m\mu$. in a Coleman Junior spectrophotometer. The correlation between optical density and number of cells/ml. culture fluid was established by comparing turbidity measurements with haemacytometer counts of cultures at different time intervals. An increase of 0·1 on the optical density scale was found to represent an average increase of $1{\cdot}6 \times 10^8$ cells/ml.

RESULTS

In replacing tomato juice by known growth factors we have followed the few analyses of this product (Cheldelin & Williams, 1942; Grossowicz, 1944), although some of the factors which proved necessary were not known to be supplied by tomato juice. The list of growth factors which was found to replace the tomato juice moiety is given in Table 2. Each of these factors appears to be indispensable for propagation of *Trypanosoma cruzi*. The partially defined medium whose composition is given in Table 2 was made up as follows: ingredients were dissolved in glass distilled water, the reaction adjusted to pH 7·8 and the medium autoclaved at 15 lb/sq.in. pressure for 20 min; the components, marked by (*) in Table 2, were dissolved separately, sterilized by filtration through Seitz pads, and added aseptically to the cooled medium.

Penicillin (60 μg./ml.) and streptomycin (100 μg./ml.) were included to decrease the risk of bacterial contamination.

Growth of Trypanosoma cruzi *on the partially defined medium.* The rate of growth and the final yield of *Trypanosoma cruzi* on the new medium is equivalent to that previously described (Citri & Grossowicz, 1954*a*) for the TJ medium and compares favourably with that obtained on undefined empirical media commonly employed for cultivation of this parasite (Table 3).

Table 2. *Composition of the partially defined medium*

		Growth factors replacing tomato juice in TJ medium			
Basal medium	g./l.		μg./ml.		μg./ml.
NaCl	4·0	*p*-Aminobenzoic acid	0·1	Riboflavin*	1·0
$Na_2HPO_4.12H_2O$	3·0	Biotin	0·2	Thiamine*	1·0
KH_2PO_4	0·5	Choline	3·0	Cobalamin	0·0001
Casein hydrolysate (enzymic)†	10·0	Folic acid	2·5	Ribonucleic acid	80
Crystalline serum albumin*	2·0	Inositol	150·0	Cytidylic acid	20
Glucose*	2·0	Nicotinamide	15·0	Creatine	20
	mg./l.	Pyridoxin	2·0	Creatinine	20
Haematin*	10·0	Pyridoxal	2·0		
Tween 80	10·0	Pyridoxamine	2·0		

* Seitz-sterilized and added aseptically to the autoclaved medium; final concentration as indicated above.

† A product of Bios Laboratories Inc., purified by adsorption on charcoal at pH 4·5.

Table 3. *Comparison of yields of* Trypanosoma cruzi *grown in different media*

Type of medium	Essential blood components	Supplemented with	Final yield cells/ml. $\times 10^7$	Authors
Semisolid	Serum	Meat extract	3·0	Adler, 1934
Diphasic	Erythrocytes and serum	Liver extract	7·0	Chang, 1947
Diphasic	Heated haemoglobin	Undefined factors	1·2	McRary, Noble & Tondevold, 1952
Diphasic	Erythrocytes coagulum	Undefined factors	0·1	Little & SubbaRow, 1945
Liquid	Haemin and crystalline serum albumin	Tomato juice	5·0	Citri & Grossowicz, 1954*a*
Liquid	Haemin and crystalline serum albumin	Defined factors (see Table 2)	5·0	present paper

Trypanosoma cruzi has now been carried on the partially defined medium through twenty-seven serial subcultures covering a period of one and a half years. Serial transfers were made at 3-weekly intervals, although the cells remained viable and motile for much longer. As no diminution in growth nor any other anomaly in development of the flagellate was detected during this period, it seems permissible to conclude that the present medium satisfies all the nutritional requirements of *T. cruzi*.

The possibility that the excellent growth described was due to the selection of a less exacting variant, which occurred during the intermediate stages of the present work, was next considered. Washed cultures of *Trypanosoma cruzi*, never previously grown in the absence of blood or serum, were sown into the present medium. The resulting growths were equal to those obtained with the strain which had been cultivated for over 2 years without blood; no decrease in growth was found in serial subcultures designed to eliminate any carry-over of the original medium.

Growth of other Trypanosomidae on the partially defined medium. Forty-three strains belonging to the family Trypanosomidae were tested for their ability to grow on the new medium; the results are summarized in Table 4.

Table 4. *Growth of various Trypanosomidae on the partially defined medium*

Species	No. of strains tested	Average count* (cells/ml.) $\times 10^7$	Remarks
Leishmania tropica	31	3·2	Rate of growth varies for different strains
L. infantum	4	—	Died off on second subculture
L. sp. (from reptiles)	4	2·6	Two strains died off on second subculture
Trypanosoma lewisi	1	—	This strain required added RBC on Adler's medium (Adler, 1934)
T. cruzi	1	5·0	—
T. acomys	1	5·1	—
Herpetomonas culicidarum	1	9·6	Visible turbidity in 24 hr.

*Where numbers are stated, no change in rate of growth or final count was observed during 10 serial transfers made to date.

DISCUSSION

The present medium differs from other media hitherto employed for serial subculture of haemoflagellates both in its physical and in its chemical constitution. Like the TJ medium previously described, it is free from insoluble constituents such as the agar included in most media (Adler & Theodor, 1926; Chang, 1947; Johnson Tobie & Rees, 1948; Seneca & Henderson, 1951; McRary, Noble & Tondevold, 1952), or erythrocyte coagulum (Little & SubbaRow, 1945, Little & Oleson, 1951). Despite elimination of all particulate components, optimal physical conditions are maintained by the simple device of incubating the culture tube sloped at an angle of 15°. The effect of the surface/volume ratio on the growth of various species of Leishmania, *Trypanosoma cruzi* and *Herpetomonas culicidarum* has already been recognized (Chang, 1947; Cowperthwaite, Weber, Packer & Hutner, 1953), and it cannot be attributed solely to improved aeration of the culture, since shaking or bubbling does not give similar results. Investigation of the physical factors conditioning growth of these flagellates may be greatly facilitated by making use of the liquid medium described above.

In replacing tomato juice by known growth factors, the chemical composition of the medium has become more defined. Surprisingly enough, the particular requirements of blood flagellates thriving on this medium proved to be much simpler than anticipated (Little & Oleson, 1951; Hamilton, Hutner, Provasoli, 1952; McRary *et al.* 1952; Kidder, 1953). A discussion of the total growth requirements of these organisms has obviously to be postponed until a minimal and completely defined medium is devised. Experiments now in progress indicate that the commercial casein digest included in the present medium may be replaced by a mixture of amino acids, with higher concentrations of some of the growth factors already recognized.

Another point which requires further study is the need for serum albumin.* The function of albumin in the present medium may perhaps be fully explained by its capacity to serve as a detoxifying vehicle of an essential lipid, namely oleic acid (Citri & Grossowicz, 1954*b*). The composition of the present medium may serve as a basis for the study of the nutritional requirements of the other Trypanosomidae whose growth it can support. Apart from its nutritional implications, the new medium provides suitable conditions for evaluation of trypanocidal agents,where defined composition and photometric growth determination is of advantage. The medium may also serve as a rich, convenient and less complicated source of cells for metabolic, cytochemical and antigenic studies.

This paper forms part of a Ph.D. Thesis presented by N. Citri to the Hebrew University, Jerusalem.

Our thanks are due to Prof. S. Adler (Jerusalem) for the strains employed in this work, and for his constant interest in the progress of the present investigation.

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* See p. 278 addendum.

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ADDENDUM

That albumin *per se* is not essential for growth of *T. cruzi* is now evident from the fact that 21 serial passages without albumin have been successfully carried out in the partially defined medium.

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The Preferential Synthesis of β-Galactosidase in *Escherichia coli*

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SUMMARY: The induced formation of β-galactosidase in relation to overall protein synthesis in *Escherichia coli* was examined under conditions where the enzyme inducer was required for growth. There was, initially, a substantial production of β-galactosidase without any detectable increase in total cell protein. This phenomenon has been termed 'preferential' β-galactosidase synthesis. The occurrence of preferential β-galactosidase synthesis indicates that β-galactosidase formation is a discrete event in cellular metabolism, and is not necessarily geared to overall cell growth. The data obtained also suggest that the carbon source(s) for preferential β-galactosidase synthesis might be of endogenous origin.

It has been amply demonstrated (e.g. Baron, Spiegelman & Quastler, 1953; Knox & Pollock, 1944; Stephenson & Stickland, 1933) that enzyme formation can occur in non-viable cells, or under conditions where environmental limitations are placed on cell proliferation. However, while these demonstrations dissociate protein formation from cell division, they do not indicate the relationship between the formation of one protein and that of other cell proteins. The work of Monod, Pappenheimer & Cohen-Bazire (1952), with *Escherichia coli*, suggests a close integration between β-galactosidase formation and the formation of other cell proteins. They found that when β-galactosidase formation was induced during growth under conditions where the enzyme was not essential for the growth of the culture, the enzyme was formed as a constant proportion of the total protein synthesized by the culture. However, earlier observations (Lester, 1951) indicated that under other conditions, where β-galactosidase was required for growth, a constant proportionality between the formation of β-galactosidase and total cell material was not apparent. A similar situation has been noted by Benzer (1953). It is the purpose of this paper to examine further the relationship between the synthesis of β-galactosidase and that of overall protein synthesis in *E. coli*.

METHODS

Strains and medium. The strains used in these experiments were *Escherichia coli*, strain W-1485, which has no growth factor requirements and is non-lysogenic, and *E. coli* strain Y-Mel_1^- which is unable to utilize melibiose for growth. Both strains were derived from *E. coli* strain K-12. A defined salts medium (Rickenberg, Yanofsky & Bonner, 1953) was used and the sugars were autoclaved separately and added after the salts solution was cooled. The cultures were grown under forced aeration at either 30° or 37°, as will be indicated.

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Preparation of cell extracts. Organisms were harvested by centrifugation, washed twice with 0·9% (w/v) NaCl solutions, and suspended in 0·05 M-sodium phosphate buffer at pH 7·0. Cell suspension (5–6 ml.) and about 3 g. Ballotini glass beads were agitated for 20 min. in a cell disintegrator (Mickle, 1948), kept at 2–3°. The disrupted cell suspensions were cleared of cell debris by centrifugation at 20,000 *g* for 1 hr. Supernatant solutions were assayed for β-galactosidase activity and protein.

Protein and β-galactosidase assay. The protein content of the extracts was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with Armour plasma fraction V as a reference standard.

β-Galactosidase activity was determined by a modification (Rickenberg *et al.* 1953) of the method described by Lederberg (1950). The reaction was carried out in a volume of 3·0 ml. with a final concentration of 0·001 M-*o*-nitrophenyl-β-galactoside (ONPG) and 0·05 M-sodium phosphate buffer (pH 7·0). After incubation at 37° for an appropriate time, depending upon the activity of the extracts, 2·0 ml. of M-K_2CO_3 were added to stop the reaction and to develop the colour of the *o*-nitrophenol released. The amount of *o*-nitrophenol released was measured in a Klett-Summerson colorimeter using the 420 mμ. (blue) filter; the values obtained were calculated for a 10 min. incubation period.

RESULTS

Previous experiments (Lester, 1951) showed that when cells were cultured in a medium containing glucose + lactose appreciable amounts of β-galactosidase were formed during the growth lag between the end of growth on glucose and the onset of growth on lactose. However, β-galactosidase synthesis was measured as a function of time, and its relationship to the overall increase in protein was not determined. The following experiment was carried out to examine enzyme formation as a function of total protein synthesis. Glucose-grown W-1485 cells were inoculated into 3·0 l. medium containing 0·03% (w/v) glucose + 0·2% (w/v) lactose and the culture incubated at 30°. Samples of known volume were withdrawn at intervals, and the cells harvested in the cold and extracted. The extracts were assayed for protein content and β-galactosidase activity.

The relationship between overall protein formation and β-galactosidase synthesis is illustrated in Fig. 1. It can be seen that during the glucose phase of growth (0–5·5 hr.) little enzyme was formed. Then, during the diauxic lag (5·5–7·0 hr.), when no increase in total protein was detected, a sharp increase in β-galactosidase activity was observed. When growth resumed, on lactose, β-galactosidase was formed as a constant proportion of overall protein synthesis. Here, in the presence of the same inducer, the relationship between β-galactosidase formation and overall protein formation differs considerably. For an equal amount of β-galactosidase, the net protein formed (if any) during the lag phase does not nearly correspond to the net protein formed during exponential growth. This formation of β-galactosidase during the lag phase is termed 'preferential' synthesis.

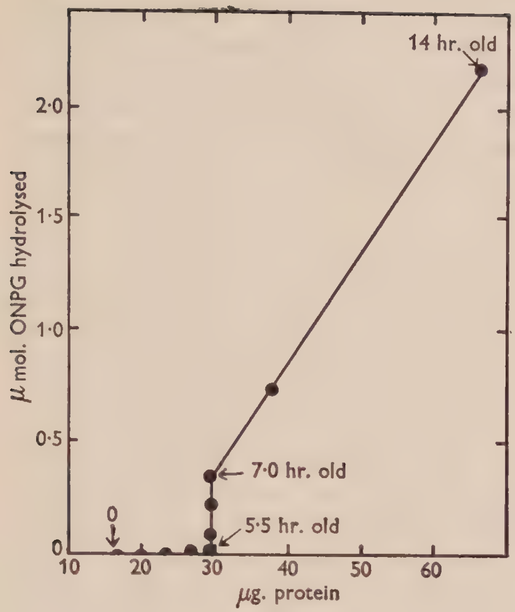

Fig. 1

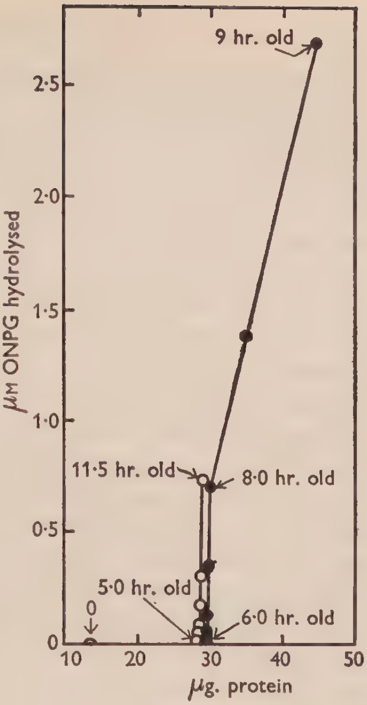

Fig. 2

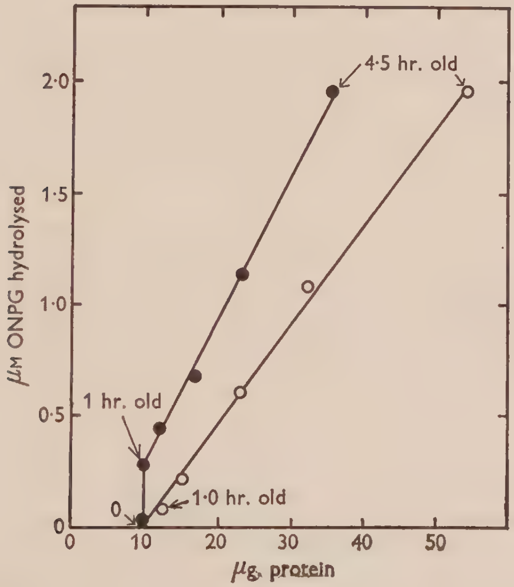

Fig. 3

Figs. 1–3. Relationship between protein and β-galactosidase activity. The values given are for the extract obtained from 1·0 ml. of culture. The numbers in the figures indicate the age of the cultures. Fig. 1. *Escherichia coli* strain W-1485 cultured in a glucose + lactose medium. Fig. 2. *E. coli* strain W-1485 (●) and strain Y-Mel_1^- (○) cultured in a glucose + melibiose medium. Fig. 3. *E. coli* strain W-1485 cultured in lactose (●) and in maltose + lactose (○) media.

The formation of β-galactosidase in the absence of overall cell growth poses a question concerning the sources of energy and carbon for β-galactosidase synthesis during the diauxic lag. Is the low level of β-galactosidase activity found during growth on glucose adequate for the utilization of lactose for β-galactosidase formation? Or does residual glucose or some endogenous material yield sufficient energy and carbon for the preferential synthesis of β-galactosidase? In order to examine this question use was made of *Escherichia coli* strain Y-Mel$_{\mathrm{I}}^{-}$.

Melibiose has been shown to be an excellent inducer of β-galactosidase formation, and cells grown on melibiose exhibit 1·5 to 2·0 times as much β-galactosidase as those grown on lactose (Monod, Cohen-Bazire & Cohn, 1951; Lester & Bonner, 1952). Strain Y-Mel$_{\mathrm{I}}^{-}$ cannot utilize melibiose, but when grown in the presence of melibiose and certain utilizable carbon sources, it will form β-galactosidase. Just as in strain W-1485, glucose inhibits β-galactosidase formation in strain Y-Mel$_{\mathrm{I}}^{-}$. Consequently, when strain Y-Mel$_{\mathrm{I}}^{-}$ is grown on a medium containing glucose + melibiose, β-galactosidase should not be formed unless glucose or some form of endogenous material is available for β-galactosidase formation. Since strain W-1485 exhibits a longer diauxic lag with glucose + melibiose than with glucose + lactose it was also of interest to determine whether preferential synthesis of β-galactosidase might also be more pronounced. Therefore, the protein content and β-galactosidase activity of strains Y-Mel$_{\mathrm{I}}^{-}$ and W-1485 were examined during growth in a glucose-melibiose medium.

Using glucose-grown strains W-1485 and Y-Mel$_{\mathrm{I}}^{-}$ as inocula an experiment similar to the one previously described was carried out. The concentrations of sugars used were 0·03 % (w/v) glucose + 0·1 % (w/v) melibiose. The relationship between total protein and β-galactosidase is shown in Fig. 2. The results obtained in the experiment with strain W-1485 resemble those observed on glucose + lactose. In this case the duration of the diauxic lag was about 2·0 hr., and a larger amount of β-galactosidase was produced during this period while no appreciable protein formation was observed. During subsequent growth on melibiose the enzyme was produced as a constant proportion of total protein synthesis. It may be noted that β-galactosidase forms a larger proportion of the protein synthesized than in the case of lactose, which is in accord with the aforementioned observations that melibiose is a better inducer of β-galactosidase formation than lactose.

When strain Y-Mel$_{\mathrm{I}}^{-}$ was used, β-galactosidase formation began when growth and overall protein formation ceased. Although the enzyme was formed at a slower rate than with strain W-1485 (requiring 6·5 hr. to reach the same activity as reached by strain W-1485 in 2·0 hr.) no growth was observed. Since strain Y-Mel$_{\mathrm{I}}^{-}$ was unable to utilize melibiose for growth, the β-galactosidase presumably was synthesized from some carbon source other than the inducer.

The experiments presented above argue against the utilization of the inducer as a carbon source for preferential β-galactosidase formation. However, the question of the exogenous or endogenous nature of the source of material for β-galactosidase formation remains. When maltose-grown cells are used to inoculate a medium containing maltose + lactose, maltose can be immediately utilized as a carbon and energy source, and β-galactosidase would not be required for the synthesis of cell protein. On the other hand, when maltose-grown cells are used to inoculate a medium containing only lactose as a carbon source, β-galactosidase will be required for cell growth. The use of a well washed inoculum decreases the possible presence of such exogenous materials as might have been present under diauxic conditions. Strain W-1485 was grown on medium containing 0·1 % (w/v) maltose, the organisms harvested after growth had ceased, and washed three times with saline. A concentrated suspension of maltose-grown organisms was used to inoculate media containing 0·2 % (w/v) lactose + 0·2 % (w/v) maltose, and 0·2 % (w/v) lactose alone. The sampling and culture conditions were as described earlier, except that the incubation temperature was 37°. The relationship between total protein and β-galactosidase activity of the extracts is illustrated in Fig. 3. It may be noted here that protein synthesis began almost immediately in the culture where maltose + lactose were present. Where lactose only was present no increase in protein was observed during the first hour of culture. When maltose + lactose were present, β-galactosidase was formed as a constant proportion of total protein synthesized. This is in agreement with the results obtained by Monod *et al.* (1952) under similar conditions.

When lactose alone was present β-galactosidase formation again took place without any detectable increase in protein. Since washed cells, which had ceased growing on a medium limiting in maltose, were used as inoculum, and since the utilization of the inducer for the preferential synthesis of the enzyme was indicated as being unlikely by the previous experiment, it is reasonable to assume that the materials for preferential β-galactosidase synthesis were of endogenous origin.

DISCUSSION

The experiments described show that, under conditions where β-galactosidase is required for growth, large amounts of β-galactosidase (approximately one hundred times the basal activity) can be produced without any detectable increase in total cell protein. Following the phase of preferential synthesis the relationship between total protein formation and β-galactosidase formation is constant; such 'proportional' synthesis is also observed under conditions where the inducer is not required for growth. Since proportional β-galactosidase synthesis occurs when the culture is actively growing, this type of synthesis would seem to be a reflexion of growth conditions rather than of a basic characteristic of protein synthesis. The preferential synthesis of β-galactosidase appears to be largely independent of the synthesis of other cell proteins, and might indicate the absence of common pathways for the synthesis of proteins in *Escherichia coli*. Thus, the formation of β-galactosidase might well be, primarily, a discrete synthetic event. These experiments strongly indicate the utilization of endogenous material for preferential β-galactosidase synthesis; however, they do not permit one to ascertain the nature of these materials. Monod & Cohn

(1953) and Rotman & Spiegelman (1954) found, under conditions where the inducer was not required as a carbon source, that pre-existing cell proteins did not appear to serve as precursors of β-galactosidase. Consequently, the possibility of protein interconversion in preferential β-galactosidase synthesis might seem unlikely. However, the conclusions drawn from experiments in which the inducer is not required for growth do not necessarily obtain for preferential synthesis, which is observed when the inducer is required for growth. The exclusiveness of preferential β-galactosidase synthesis, and the induction of β-galactosidase formation by the melibiose-less mutant, might argue for the utilization of endogenous material for preferential synthesis.

One of the authors (H.V.R.) is a Predoctoral Fellow of the National Institutes of Health.

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Lampropedia hyalina Schroeter 1886 and *Vannielia aggregata* n.g., n.sp., with Remarks on Natural and on Organized Colonies in Bacteria

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SUMMARY: Two bacteria, *Lampropedia hyalina* and *Vannielia aggregata*, are described, which form characteristically shaped aggregations. The differences between artificial, natural and organized colonies are discussed.

Lampropedia hyalina was observed in April 1951 in liquid manure of brown colour from a dairy farmyard on Madingley Road, Cambridge. After 1 day's standing in the laboratory loops-full from the surface contained the following organisms: great numbers of *Spirillum* spp. of various sizes in part distorted; *Polytoma uvella*; small colourless flagellates; bacteria in chains, often with longitudinal division so that two parallel rows originated; a large bacterial rod in chains (Lineola?); motile sarcinas, cells 2–3 μ. in diameter, arranged in cubiform clusters, the smaller ones being more active; and bacterial aggregates with growth in one plane though not always flat, some of them swimming in a sort of unbalanced swaying movement.

It is this last organism which was taken into culture and is believed to be identical with Schroeter's (1886) *Lampropedia hyalina*, in spite of its initial motility which was lost on culturing. If there was not perhaps a confusion with the Sarcina (which is not very likely, but cannot be checked any more) Lampropedia would be in synonymy with *Pedioplana haeckeli* Wolff (1907) which recalls a flagellate Lampropedia ('erinnert an eine mit Geisseln versehene Lampropedia'). This had been found in rotting-portions of mangel-wurzels or swedes, and such were also present in the dung-heap from which the manure for this investigation was taken. Lampropedia was first observed in muddy water and probably given its name because under the microscope it appeared like glistening tablets. Lampropedia is one of the relatively few bacteria which can be recognized under the microscope. It does not seem to be rare and may occur in enormous numbers. *Gonium hyalinum* Ehrenberg (1838), *Merismopedia hyalina* Kützing (1845–71) and *Micrococcus hyalinus* Migula (1897–1900) were probably identical with Lampropedia. The species may be planktonic, but is more often found as a film covering dirty mud or the overlying water. Kolkwitz (1909, p. 90) records it from several of the lakes near Berlin which are polluted by waters from neighbouring habitations, and considers it to be a mud organism.

Plating of the original material failed to produce colonies of Lampropedia, owing to the overwhelming number of other bacteria, but single tablets transferred with a capillary pipette to soil-water cultures with starch or a wheat grain on the bottom gave rise to mixed cultures. Lampropedia tablets appeared after a few days in great numbers in the surface film and could be transferred again with a capillary to agar plates where they were streaked out with a loop. After one day at room temperature a few isolated colonies were

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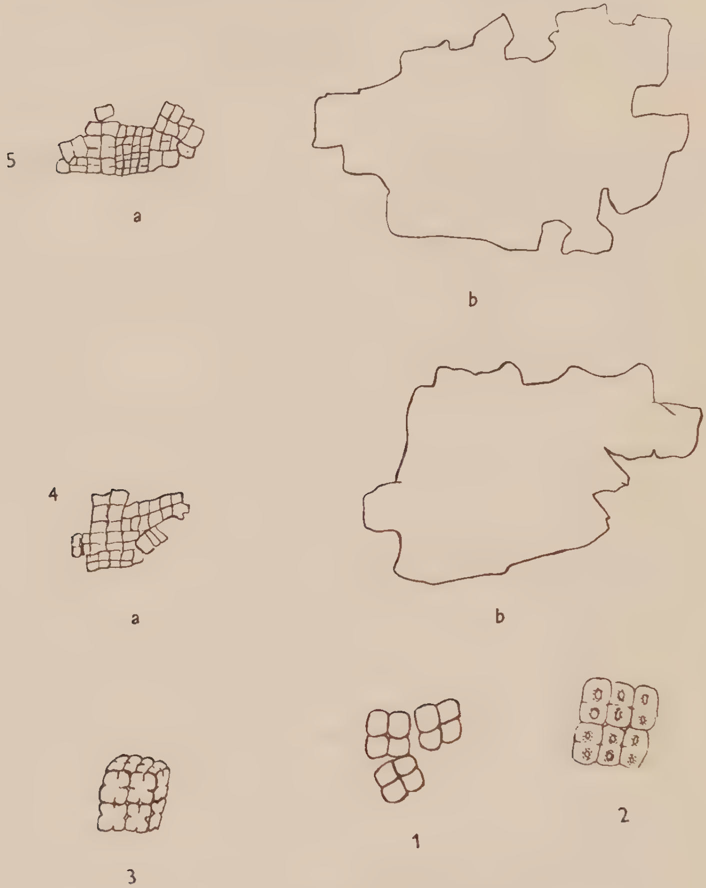


Figs. 1–5. ***Lampropedia hyalina.***

Fig. 1. Group of three tetrads. × 3000. Fig. 2. Small plate, vitally stained with neutral red. × 3000. Fig. 3. Colony of a sarcina associated with Lampropedia at the original habitat. × 3000. Figs. 4*a*, *b* and 5*a*, *b*. Two examples of: *a*, small initial group, a fragment of a previous growth, transferred to a new agar plate; and *b*, the same in outlines after 1 day at room temperature. × 1500.

observed on agar with 0·1 % sodium acetate, 0·2 % Difco yeast extract and 0·1 % Difco tryptone. It was found later that acetate was not necessary to obtain growth of this bacterium, but served only to neutralize the otherwise slightly acid medium. Lampropedia multiplies only in neutral or weak alkaline media. By repeating the plating procedure, pure cultures were obtained. They multiplied still better at higher concentrations of nutrient substances, for instance 0·5 % Difco Bacto peptone or Difco tryptone + 0·5 % Difco yeast extract, pH 7, or similar mixtures as used for other bacteria. The most luxuriant growth was observed on the medium devised for *Caryophanon latum* (Pringsheim & Robinow, 1947) and composed as follows: sodium acetate 0·1 %, beef extract 0·1 %, yeast extract 0·2 %, agar 1·5 %.

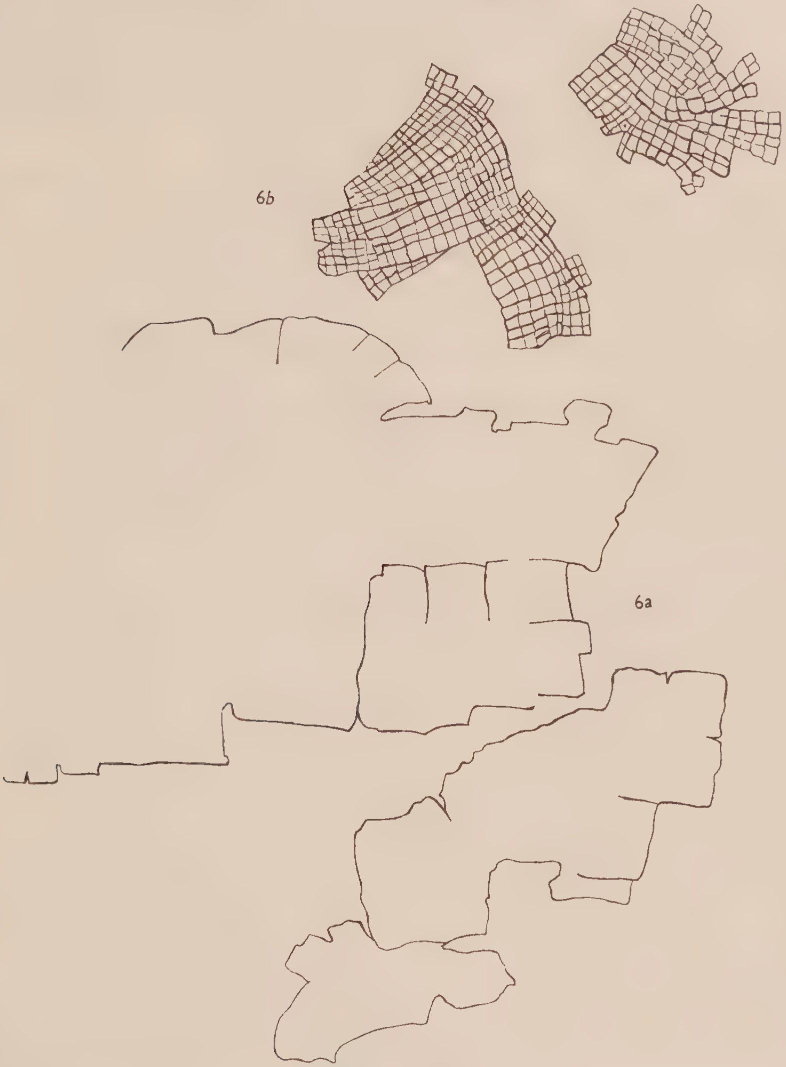


Fig 6. ***Lampropedia hyalina.*** ***a,*** **outlines of part of a 3-day old growth on agar plate;** ***b,*** **details with cell margins shown.**

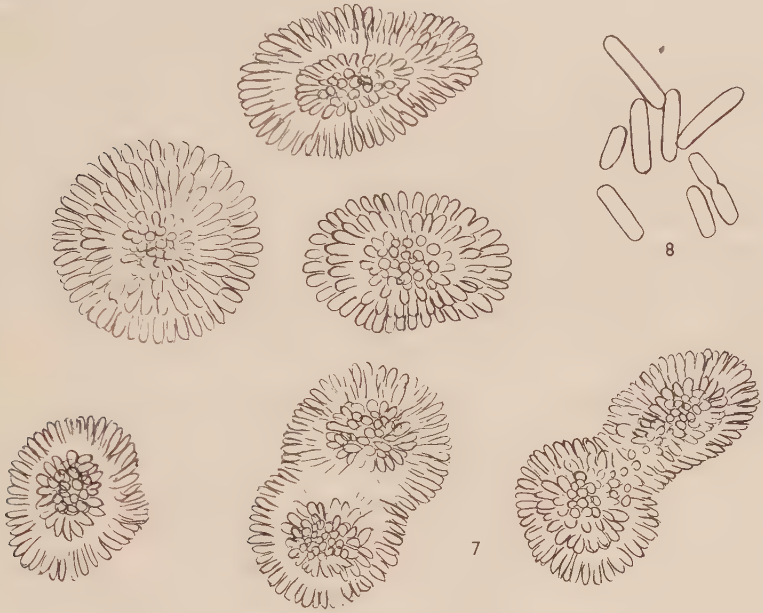
The growth on agar slopes may be very luxurious when the medium has been neutralized and is damp enough. The development of the small squarish fragments transferred to the wet agar surface with a loop can best be followed on Pl. 1 and in Figs. 4 and 5. The ensuing sheets extend while growing in all directions, their margins slipping over the agar. Only when there is more friction, either because the watery surface film has evaporated or because the sheets meet, do they get wrinkled and folded or roll up at the borders (Pl. 1, fig. 5). There is never a diffuse growth of single or paired cells. In liquid media, the growth adheres to the glass wall and eventually falls to the bottom or reaches the surface. Only in the latter case does a thriving development occur. The cell aggregates are then not readily wetted, and their upper surface appears to grow less than the lower, or there is some shrinking so that the cell plates curve and attain irregular shapes (Pl. 1, fig. 6).

Vannielia aggregata n.g., n.sp. was found with Spirogyra in June 1953 in a jar of putrifying mud from Coe Fen, a piece of pasture land in Cambridge with ditches and trees. Ecologically the habitat is characterized by a tendency towards polytrophic conditions as borne out by the occurrence of various Euglenaceae, mainly *Euglena viridis*, *Phacus pyrum* and *Eutreptia viridis*, of *Spirillum*, *Chlamydomonas*, and *Eudorina* spp., *Spirulina albida* and *Sphaerotilus natans*.

Pink patches appeared on the edge of the fluid away from the light, composed of motile organisms which re-assembled after mechanical disturbance. They turned out to be clusters of radiately arranged bacterial rods. These clusters were ovoid to spherical in shape and 15–17 μ. in diameter, rotating with the help of flagella, one to each cell, and markedly phototactic. The roundish clusters were not very regular, pinkish in the centre, but not appreciably coloured in the peripheral half of the radius. The individual rods were about 1 μ. across, 4–6 μ. long, and Gram-negative. After drying, methylene blue and particularly fuchsin stained them well. Indian ink and nigrosin failed to demonstrate a mucilaginous area around the colonies which might have explained the keeping in place of the individual rods in the clusters. Living clusters suspended in the original fluid and enclosed in a hollow slide sealed with vaseline and a cover-slip, remained alive for several days, swimming about all the time, while in hanging drops they soon ceased moving, and were overgrown with colourless bacteria.

Attempts at cultivation started with material from one of the reddish patches sucked into a fine pipette and blown into soil-water tubes with various kinds of putrifying material, of which small pieces of cheese proved best. After 4 days, pink spots appeared in the test-tubes remote from the light of a north window. Next day, there was a rose-coloured little cloud just above the soil, and from there a whitish band stretched upwards. On the sixth day, plentiful colonies were seen forming whitish pink clouds which reached the surface, and soon after a reddish lilac aggregation formed in the uppermost portion of the fluid. After a week *Chlamydomonas* spp. appeared and reduced the reddish organism more and more. By repeatedly diluting and subculturing, the suspension of *Vannielia aggregata* could be maintained in cheese + soil tubes for several weeks, although not every culture was successful, owing to the admixture of green flagellates. Transfer of individual colonies with a capillary pipette into new putrifaction tubes was not successful.

After several failures pure cultures were obtained by mixing drops of red motile Vannielia clusters into test-tubes with Difco nutrient agar, preferably diluted to one half with watery agar, and repeating this procedure several times. In the beginning, red and green colonies appeared after a week (north window, June 1953) in the more diluted suspensions, far enough from one another to be picked up after breaking the tubes and removing the agar columns. Heavily inoculated subcultures free of other bacteria showed maximum growth at about 5 mm. from the agar surface, but small reddish colonies filled the agar down to the bottom.



Figs. 7, 8. ***Vannielia aggregata.***

Fig. 7. One colony normal and spherical, three irregular and longish, two in a state of division. The darker inner portion of the colonies only appears pinkish. × 1500.
Fig. 8. Individual cells. × 3000.

The strain could be maintained in stab cultures, and growth occurred also in peptone yeast extract liquid media and soil + cheese + water cultures. No well-organized spherical clusters were, however, found in these, the nearest approach being small aggregations of cells only partly radially arranged. Single cells swimming with extraordinary rapidity were in the majority.

It is therefore not absolutely certain that the true Vannielia was isolated and, under unnatural conditions, aggregated in a more irregular way, though this seems to be the best explanation.

The genus *Vannielia* is obviously a member of the Athiorhodaceae and ought to be grown in conditions more similar to those at the habitat. This would be worth while, since formation of definite colonies is only known in the Thiorhodaceae (*Thiopedia*, *Thiodictyon*) but not in the sulphur-free purple bacteria.

We speak of colonies of bacteria when small clusters of cells, often derived from one original cell, develop by binary fission into heaps of thousands or millions of cells and become visible to the naked eye. These are artificial colonies rarely found in nature. With other organisms, for instance flagellates and algae, but also with metazoa like Coelenterata and Bryozoa, and even with insects and mammals, the term colony is used in a different way, viz. for individuals living together under natural conditions and forming a community somehow biologically connected to be components of a whole. There are in bacteria not many instances of such communities.

Artificial colonies of bacteria growing in the habitual manner on semisolid media show of course very often a characteristic arrangement which is helpful in identification, for instance the curls of *Bacillus anthracis*, or the branched mycelium-like appearance of *B. mycoides*. But in liquid media this arrangement is lost, and nothing but chains of cells are observed. Only in very few species, as, for example, *Zoogloea ramigera*, *Sphaerotilus natans* and *Crenothrix ochracea*, the cells are kept together by mucilage or sheaths to constitute well-recognizable structures. Others remain connected after cell division, without a special enveloping substance being recognizable, for instance *Sarcina*, *Thiopedia*, *Thiodictyon* spp. To this category belongs Lampropedia, remarkable for the great number of cells which may form such natural colonies, which are also produced on nutrient agar, so that artificial colonies are not much different from them.

Vannielia is of another type and more reminiscent of organized colonies as found in flagellates like Chrysophyceae (*Synura*), Craspedomonadaceae (*Sphaeroeca*) or Volvocaceae (*Pandorina*) in being composed of cells connected in the centre by their posterior ends and swimming with the help of flagella emerging from the individuals into the surrounding water.

In all these instances a unifying impulse must be supposed to correlate the action of the flagella; no regulated forward locomotion would otherwise be possible. *Pelosphaera rotans* (Lauterborn, 1915, p. 420, Pl. II, figs. 18, 19) with a diameter of the colonies of 15–40 μ. must be similar to Vannielia. According to Koppe (1923, p. 634), it occurred in smelly water of low oxygen content underneath a Lemna cover. This would be in keeping with the identity of Pelosphaera and Vannielia. But no colour is mentioned in the description, and the size is larger than that of Vannielia, so that identity is not probable.

The arrangement of the cells is different in *Sarcina*, *Planosarcina* (Migula, 1900) and *Pedioplana* (Wolff, 1907) species, but these colonies also are organized, swimming in a well regulated way with their flagella pointing in one direction, comparable to the colonies of Gonium among Volvocaceae.

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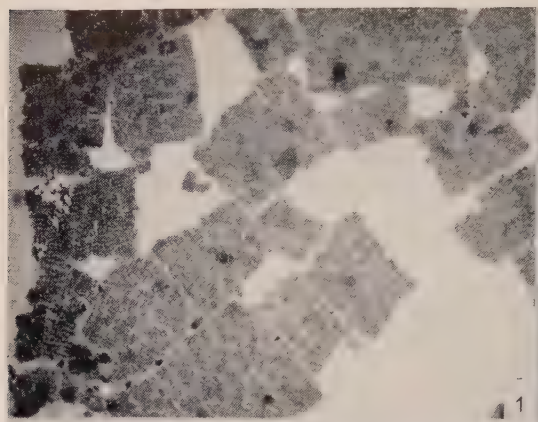

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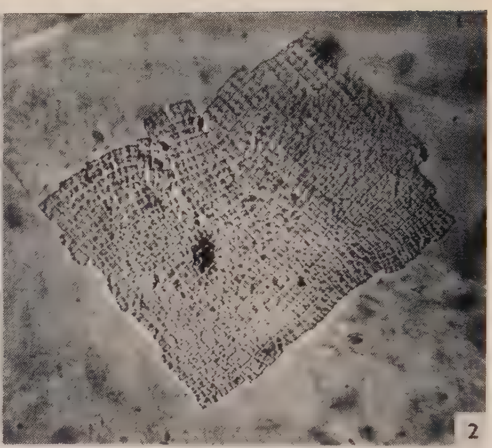

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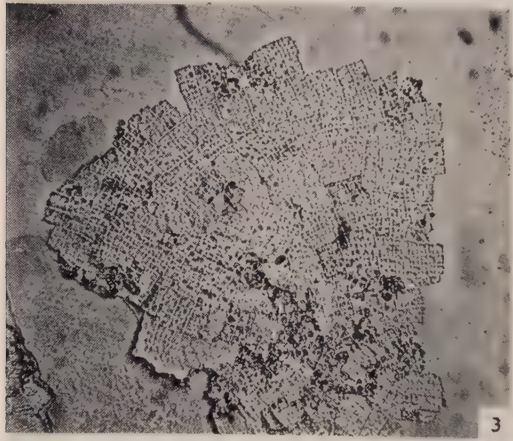

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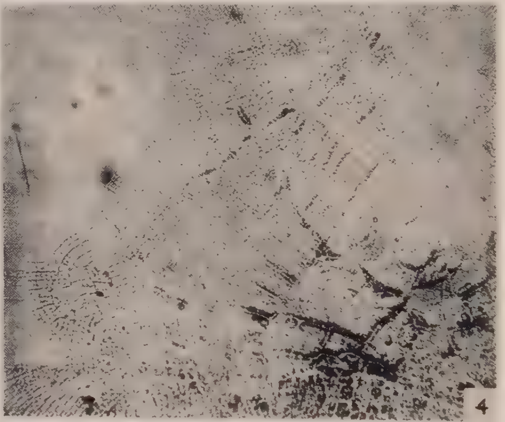

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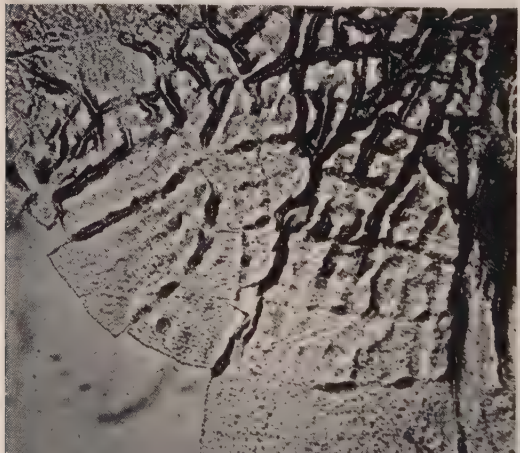

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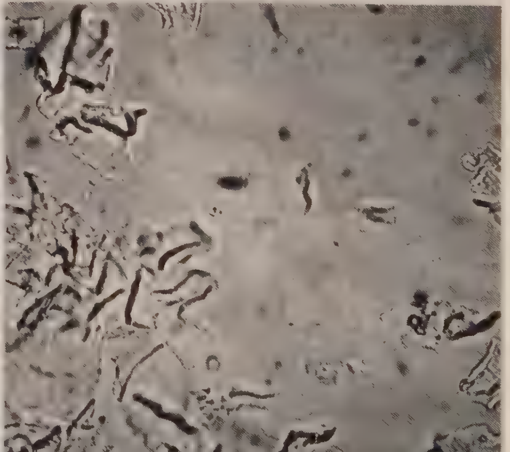

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E. G. Pringsheim—*Lampropedia* and *Vanniella* species. Plate 1 (*Facing p.* 291)

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EXPLANATION OF PLATE

Fig. 1. Colonies from agar, stained with fuchsin.

Fig. 2. A living, squarish colony.

Fig. 3. Older colony, uniform growth no longer maintained, with individual squares distinguishable.

Fig. 4. Large colony on agar; arrangement of cell-rows partly fan-like. Detachment from agar beginning along straight ridges conditioned by growth parallel to the surface of the medium.

Fig. 5. Outer portion of an older growth on agar, inner parts folded and to some degree lifted from the surface of the medium by growth pressure and friction.

Fig. 6. Colonies from surface of liquid medium, some of them curled and dark from adhering air.

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Hayward, A. C., Hale, C. M. F. & Bisset, K. A. (1955). *J. gen. Microbiol.* **13**, 292–294

The Morphology and Relationships of '*Lactobacillus*' *bifidus*

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SUMMARY: Strains of '*Lactobacillus*' *bifidus* were found to vary in morphology from large globules to more slender septate rods and filaments, often very profusely branched. Adaptation of anaerobic strains to growth under aerobic conditions is usually accompanied by a diminution in branching. The organism has little or no morphological resemblance to a true *Lactobacillus* species, but strongly resembles certain strains of *Corynebacterium*.

The systematic position of the branched Gram-positive bacterium found in the faeces of infants and described in *Bergey's Manual* (1948) as *Lactobacillus bifidus*, according to the classification of Weiss & Rettger (1934), has been the subject of considerable controversy. It is defined as a member of the genus *Lactobacillus* on the grounds that it is non-motile, non-sporing, catalase-negative, does not reduce nitrates, and ferments carbohydrates with the production of lactic and acetic acids. It will be observed that these characters are preponderantly negative, which is rather unsatisfactory. On the other hand, it differs from typical members of the genus in being an obligate anaerobe, at least on primary isolation, in exhibiting true branching, and in having a very restricted range of habitat and temperature requirement (Norris, Flanders, Tomarelli & György, 1950). Olsen (1949) considered it to be a species of *Corynebacterium*, and the generic names *Bacteroides*, *Nocardia* and *Actinomyces* have at various times been conferred upon it. In the course of a recent investigation of the morphology of *Lactobacillus* species in this laboratory (Davis, Bisset & Hale, 1955), strains of this organism were examined, and were considered to merit a separate description.

METHODS

Fourteen strains of organisms corresponding to the foregoing description were used. Of these one strain was supplied by courtesy of Dr A. T. R. Mattick, one was isolated from turkey faeces, and the remainder from faeces of newborn infants.

Primary isolations were made upon the tomato-juice agar described by Davis *et al.* (1955) in the form of plates, incubated at 37° in a McIntosh and Fildes jar. Subsequent cultures were grown in stab tubes of the same medium or in tubes of broth covered with vaseline. *Lactobacillus bifidus* is difficult to maintain in culture, and it was found advisable to subculture at intervals of 24 hr.

The organisms were examined cytologically by Hale's (1953) method for cell walls.

RESULTS

When successfully subcultured for a period of *c.* 10 days isolates of this bacterium became progressively adapted to existence in artificial medium, grew more freely and gradually lost their sensitivity to oxygen. The degree of sensitivity varied considerably in different strains, but the majority, when newly isolated, showed after 24 hr. only a faint line of growth in the lower part of the stab. Later subcultures showed a much heavier growth, extending almost to the surface of the medium. This diminution in sensitivity to oxygen was reported by Weiss & Rettger (1934) as characteristic of their *Lactobacillus bifidus*, type I.

The various types of morphology observed are illustrated in Pl. 1, figs. 1–5. Characteristically the organisms resembled corynebacteria, as described by Bisset (1949), and included branched and unbranched septate rods with expanded terminal cells (Pl. 1, figs. 1, 3). Some were filamentous, or alternatively, so profusely branched as to cease to bear any resemblance to rods; examples of both are seen in Pl. 1, fig. 2. A different morphological form appeared also in some cultures; these cells were unbranched but swollen and irregular (Pl. 1, figs. 4, 5). As the strains became adapted to artificial culture, the branched and swollen forms became less and less frequent, and the simple corynebacterium-like rods predominated. Although the observation is of necessity subjective, there appeared at all stages of culture to be a correlation between the preponderance of branched or swollen organisms and the degree of sensitivity to oxygen of the culture (cf. Friedman & Black, 1941; Prévot & Raynaud, 1955). Thus a well-adapted culture might superficially resemble species of *Lactobacillus*, but a comparison of the cytological structure of these rods (e.g. the unbranched forms in Pl. 1, figs. 1–3) and of true lactobacilli (Davis *et al.* 1955) reveals the difference very clearly.

DISCUSSION

It appears that the resemblances between '*Lactobacillus*' *bifidus* and the true lactobacilli are almost entirely negative, and do not extend to the details of morphology as these are demonstrable by cytological examination. Morphologically '*L*'. *bifidus* appears to belong to the Actinomycetales, suborder Mycobacteriineae, as defined by Bisset (1952). It is not, perhaps, quite justifiable to assign it to the genus *Corynebacterium*, but it may be permissible to speculate whether it might not have originated in an organism of this type, adapted, as so many are, to parasitism upon mucous membranes, in this case that of the vagina, thence passing to the lower gut of newborn infants, and subsequently to a wider, but still restricted, faecal habitat.

The generic title *Bifidobacterium* (Orla-Jensen, 1924) is considered to be valid and applicable to the organism studied.

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EXPLANATION OF PLATE

Figs. 1–5. *Lactobacillus bifidus*, stained by Hale's method for cell walls; ×3000. 24 hr. cultures. Figs. 1–3. Filamentous and branched forms. Figs. 4, 5. Globular forms.

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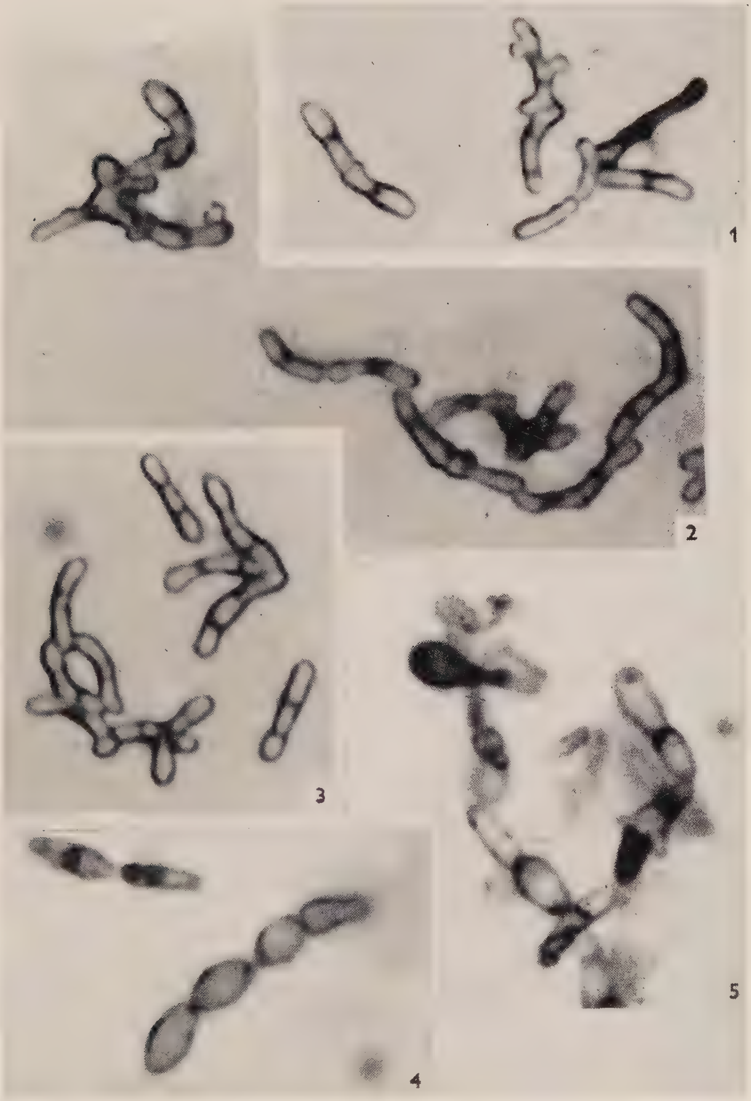

A. C. Hayward, C. M. F. Hale and K. A. Bisset—Morphology of *Lactobacillus bifidus*. Plate 1

(*Facing p.* 294)

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'Fruity' and other Mutants of the Cellular Slime Mould, *Dictyostelium discoideum*: a Study of Developmental Aberrations

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SUMMARY: Study of three mutants of *Dictyostelium discoideum*—Fruity, Bushy and Dwarf—provide specific examples of aberrant behaviour during aggregation and the construction of fruiting bodies. The proportion of initiator cells in the population and the optimal population density for aggregation have been shown to be under genetic control. In the Fruity mutant, the great increase in the number of initiator cells leads to the formation of aggregates composed of very few individuals and correspondingly tiny fruits. The morphology of the aggregates and fruits and the regulatory properties of this morphogenetic system have been examined.

Slime mould myxamoebae, otherwise typical Protista, collectively display a remarkable developmental capacity leading to the formation of organized multicellular structures (Raper, 1941; Bonner, 1944; Sussman, 1955). Aggregation, the part of the morphogenetic sequence which is of chief concern here, serves as a device to bring the hitherto independent cells into a compact mass. It begins after the stationary growth phase has been reached, the myxamoebae lengthening greatly and orienting radially. The cells then move in toward centres of aggregation, at first as individuals and later in ramified cell streams. The conical aggregate which results is transformed into an organized pseudoplasmodium which migrates over the agar surface and ultimately gives rise to a sessile fruiting body consisting of a spore mass, a parenchymatous stalk, and a basal disk.

Some insight into the mechanisms underlying the aggregative process has been gained as a result of previous investigations. The response of the outlying cells to the aggregative stimulus has been shown by a variety of means to be chemotactic (Runyon, 1942; Bonner, 1947, 1949; Shaffer, 1953; Sussman & Lee, 1954, 1955). The attracting substances, given the generic name 'acrasin' by Bonner, are produced at the aggregative centres and along the streams and are readily diffusible and highly unstable. That both the agents and the chemotactic responses are specific has been indicated in studies of mixed aggregations by cells of different species and genera (Raper & Thom, 1941) and by treatment of myxamoebae with washings obtained from heterologous aggregates and pseudoplasmodia (Shaffer, 1953).

The aggregation of myxamoebae on a washed agar distilled water substratum has been examined under conditions where the cells remained constant in number and where rigorous control over population size and density could be achieved (Sussman & Noel 1952; Sussman, 1952).

The population density (no. cells/mm.^{2}) radically influenced the number of aggregative centres appearing within populations of constant size. The population density which permitted maximal centre formation was found to be characteristic of the species employed (Sussman & Noel, 1952) and largely invariant, but it could be altered by addition to the washed agar medium of a restricted number of substances, notably including the amino acid histidine and the purine base adenine (Bradley & Sussman, 1954).

At the optimal population density, the number of centres was directly proportional to the number of cells present. Two independent experimental approaches (Sussman & Noel, 1952; Sussman, 1952) indicated that this proportionality stems from the fact that the evocation of each centre requires the presence of a special initiator cell and that, under the conditions employed, only a few cells in the population are so constituted. In the species *Dictyostelium discoideum* used in the current studies, the ratio of initiator cells to responder cells was found to be 1 : 2200.

Heritable modifications of the aggregative pattern have previously been observed (Sussman & Sussman, 1953; Sussman, 1954) in aggregateless mutants which remain as dispersed amoebae and in some fruitless varieties which can start aggregating but which cannot complete the process. The present paper deals with the aberrant behaviour of three other mutant stocks, both during aggregation and in the subsequent erection of fruiting structures. In contrast to the morphogenetically deficient mutants alluded to above, all three strains can aggregate and produce fruiting structures with viable spores. They are of particular interest in that the modifications of their aggregative patterns include changes in the optimal population density for centre formation and/or increases in the proportion of initiator cells.

METHODS

Organisms and methods of culture. Dictyostelium discoideum (Raper) strain NC-4, including the wild type and three mutants was used in this investigation. The stocks were grown routinely on SM agar (Sussman, 1951) in association with *Aerobacter aerogenes.* For routine transfers 4 or 5 spore masses were picked and spread with a few drops of 48 hr. *A. aerogenes* broth culture. For clonal platings, less than 200 spores were inoculated, each viable spore giving rise to a plaque (Sussman, 1951) after 3 days of incubation at 22°.

Washed agar medium for aggregation experiments. Twenty-five g. of Difco Bacto agar was washed on a table-top Büchner filter with 6 l. distilled water and dissolved in 1 l. water; this will be referred to as 'minimal agar'. In certain experiments streptomycin sulphate (370 units/l.) was added before pouring the plates. Streptomycin has been found (unpublished data) to aid in preventing the growth of bacteria and therefore of the myxamoebae without interfering with the developmental pattern.

Preparation of the cells and experimental procedures. The organisms were grown for about 44 hr., by which time the stationary growth phase had been reached but aggregation had not yet begun. The cells were harvested with chilled water and spun at 1000 r.p.m. for 5 min. in an International refrigerated centrifuge to remove the few remaining bacteria. After three more washings the myxamoebae were suspended in salt solution (Bonner, 1947) and counted in a haemocytometer. Dilutions of the suspension were dispensed in various sized drops on minimal agar with 0·1 ml. Mohr pipettes. The Petri dish covers were left open until the fluid had been absorbed by the agar, after which the plates were incubated at 22°. Counts of aggregative centres and fruits were made by microscopic examination after 18 and 36 hr. of incubation.

RESULTS

Isolation and characterization of the Fruity mutant of Dictyostelium discoideum

This stock, as well as the mutants described later, was encountered after ultraviolet irradiation of the wild type. The procedure used was described elsewhere (Sussman & Sussman, 1953). The mutant was recognized by its ability to produce relatively enormous numbers of tiny fruiting bodies, which property suggested the designation Fruity and the abbreviated symbol, Fty-1. The Fruity mutant differs from the wild type in certain other respects. While the growth rates are approximately equal, the mutant plaques are much smaller than those of the wild type and possess a sharper and more regular periphery. Moreover, the first fruitings of the mutant appear when the plaques are very tiny, whereas in the wild type fruiting is considerably more delayed. The mutant phenotype is very stable and no reversions have as yet been detected in the course of hundreds of clonal and mass cultivations. The spores and vegetative myxamoebae of the mutant are indistinguishable from those of the wild type by cursory microscopic observation. Detailed cytological comparisons have not been made.

Fty-1 *aggregations on minimal agar*

Fty-1 was grown and prepared for experiment as described in the Methods section. Myxamoebae suspensions were dispensed on minimal agar in volumes ranging from 0·005 to 0·1 ml. The specific dilutions were chosen in order that a constant number of cells be delivered in each sample. Previous experiments (Sussman & Noel, 1952) had shown that the area covered by the drops and, thereby, the population density of the cells, are functions of the volume delivered. In each experiment, the population densities were checked either by measurement of the areas covered or by direct determination with a Sedgewick ocular grid. After 24 hr. of incubation, the aggregative centres or the fruits (one formed per aggregate) were counted under a 16 mm. objective. To facilitate counting, the agar within the confines of each drop was lightly scored with a razor blade in a grid-like pattern; these marks served as guide lines.

Figure 1 summarizes the data obtained from three such experiments with three different population sizes, namely, 10,000, 5000, and 4000 cells. Other experiments not summarized here gave the same results. The number of centres per drop has been plotted against population density. Each point represents a mean of at least four replicate determinations. Table 1 presents numerical data bearing upon the number of centres formed at the optimal density.

Comparisons can be drawn at several points between the aggregative performance of the mutant and of the wild type (see the lower curve in Fig. 3 for reference). The most important difference lies in the capacity of the mutant to produce a great number of aggregates. Thus, at the optimal density, 10,000 mutant cells produced 423 centres for a ratio of centres to cells of 1 : 24. In contrast, 10,000 wild type formed less than five centres representing a ratio of 1 : 2200 (Sussman & Noel, 1952). Table 1 also indicates that at the optimal density the number of centres is in linear relation to the number of cells present, as is the case for the wild type.

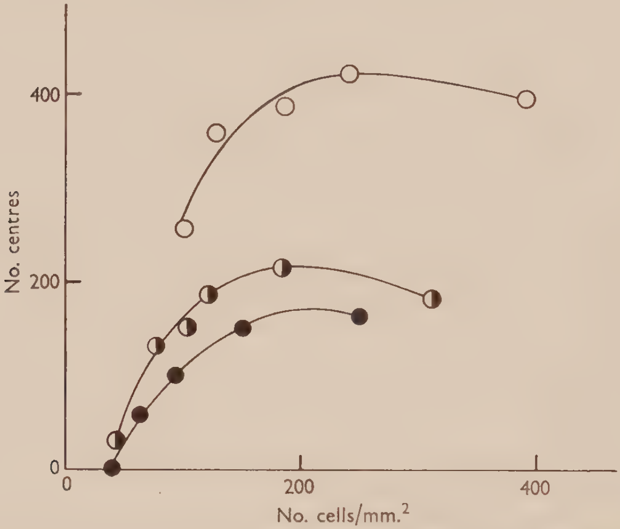

Fig. 1. The relation between centre formation and population density for Fty-1 on minimal agar. The ordinate refers to the number of centres formed/sample of 10,000 cells (upper curve ○), 5000 (middle curve, ◐), and 4000 (lower curve, ●).

Table 1. *The relation between the number of aggregative centres and the number of cells in Fty-1*

No. of cells	No. of centres	Ratio: centres : cells	Optimal density
10,000	423	1 : 24	240
6,000	250	1 : 23	180
5,000	213	1 : 24	200
4,000	168	1 : 24	210

Washed Fruity cells were dispensed on minimal agar over a range of population densities. After incubation, counts were made of aggregative centres. Densities shown in the last column were those at which maximal centre formation occurred.

The optimal densities for centre formation appear to be about the same for the two stocks. Similar decreases of the numbers of centres are observed at high population densities, although the mutant appears less sensitive in this

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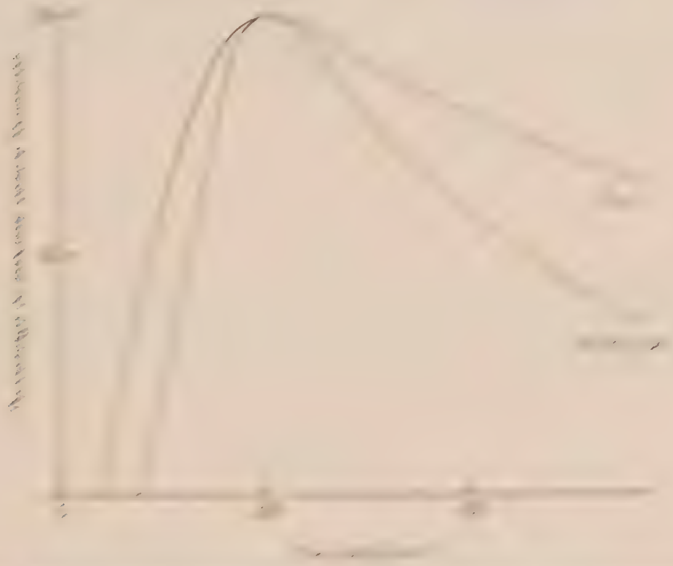

Fig. 2. [illegible]

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had led to an increase in the proportion of myxamoebae which could act as initiators. In this case no special sensitivity need be ascribed to the mutant responder cells. To test these possibilities, cell mixtures containing very small numbers of mutant cells and very large numbers of wild-type cells were examined. In such a system, the majority of responder cells would be wild type, thereby uncoupling the mutant initiator/responder system.

The cells were grown, washed and suspended in salt solution. Mixtures were prepared and dispensed on minimal agar in different sized drops so that samples, each containing 10,000 wild type and 500 mutant cells, were dispersed over a range of population densities. Control suspensions of wild type only were similarly treated. After incubation, counts were made of the centres formed. Figure 3 shows the results of a typical experiment; the points represent averages of at least ten replicate samples. In all, six experiments were performed, involving nine different mixtures. Outside of the significant increase in number of centres formed, the experimental and control curves are quite comparable. The threshold of sensitivity at low population density appeared to be governed by the nature of the responding cells. In all of the experiments the lower limit was *c.* 80 cells/mm.^{2} rather than 40 which obtains when only mutant cells are present. The optimal population density was the same. The usual diminution of centre formation at densities above the optimal was apparent, though not as marked as in wild-type populations. Rather it was more like that observed with the mutant (see Fig. 2), suggesting that this diminution depended at least in part upon the nature of the initiator cells.

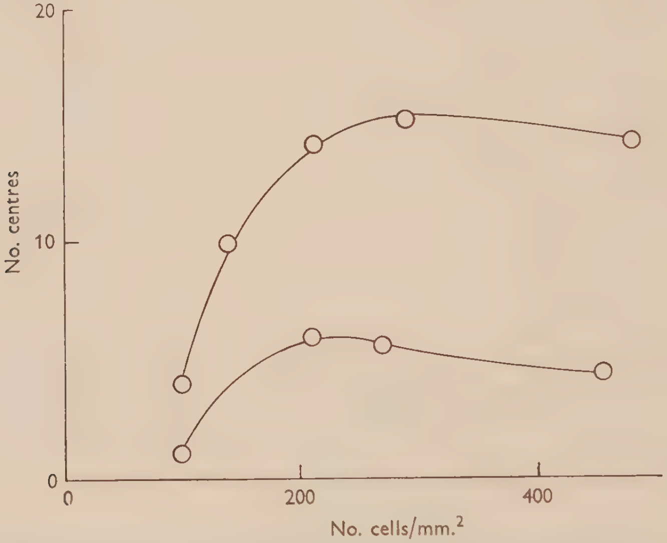

Fig. 3. The relation between centre formation and population density for *Dictyostelium discoideum* wild type (lower curve) and for a mixture of wild type and Fty-1 (upper curve).

To determine the number of centres in the mixture due only to the presence of mutant initiator cells, the centres evoked by wild-type initiators at a given population density (obtained from the control curve) were subtracted from the total number of centres of the mixture. In this manner points were obtained for a new curve which related the number of centres evoked by the Fruity initiators to the population density of the mixture. Figure 4 shows two such curves; the data were obtained from four experiments with mixtures containing 1000 Fty-1 cells (upper curve) and 500 (lower curve). In each case a plateau was reached at a density approximating 300 cells/mm². Table 2 gives the plateau values for three different numbers of mutant cells mixed with 10,000 wild type. The mean ratio of centres evoked by mutant initiators to mutant cells is seen to be 1 : 58. In another series of experiments, mixtures containing from 400 to 1200 mutant cells and 10,000 or 25,000 wild type were dispensed at densities around 400 cells/mm². Table 3 gives mean ratio values for the mutant, each representing at least four replicate determinations. The

Table 2. *Centre formation by mixtures of wild type and Fty*-1 *cells*

No. of Fty-1 cells	No. of centres at plateau	Ratio
1000	15·8	1 : 63
	15·6	1 : 64
750	11·9	1 : 63
	14·6	1 : 50
500	8·8	1 : 57
	9·8	1 : 51
	Mean	1 : 58

The washed cells were mixed in varying proportions and dispensed over a range of population densities as described in the text. Values in the second column were derived from the curves shown in fig. 4 and from others not published. ‘Ratio’ in the last column refers to the ratio of centres evoked by mutant initiators to the number of mutant cells present.

Table 3. *Centre: cell ratios for Fty*-1 *mixed with different numbers of wild type*

A		B	
No. of Fty-1 cells	Ratio	No. of Fty-1 cells	Ratio
1200	1 : 59	1200	1 : 61
1100	1 : 56	1100	1 : 59
1000	1 : 62	1000	1 : 60
900	1 : 65	900	1 : 63
800	1 : 61	800	1 : 60
700	1 : 64	600	1 : 58
600	1 : 58		
500	1 : 62		
400	1 : 64	Mean	1 : 60
Mean	1 : 61		

See text for experimental details. The values given in column A were obtained from mixtures of the mutant with 10,000 wild type myxamoebae; those in column B with 25,000. The term ‘Ratio’ refers to the ratio of centres evoked by mutant initiators to mutant cells present in the mixtures.

ratio is constant and appears to be independent of the number of wild type present or the state of dispersion of the mutant cells. The fact that the number of centres ascribed to the mutant is proportional to the number of Fruity cells (thus providing a constant ratio) and is independent of the state of dispersion of the Fruity cells in the mixture indicates that single mutant cells act to initiate the aggregative centres.

The plateaus that appear in Fig. 4 persist even at extremely high population densities. The results of one experiment with mixtures of 1000 mutant and 50,000 wild type showed that the usual ratio occurred even at a density of 1100 cells/mm.^{2} and only at 1800 was a significant diminution of centre formation observed.

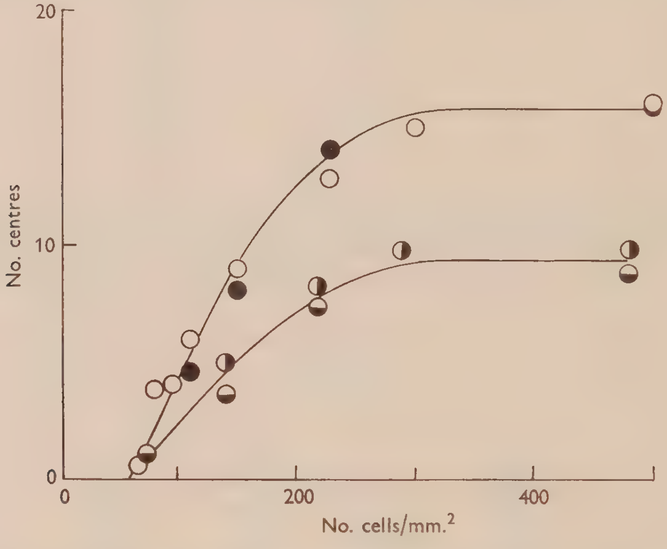

Fig. 4. The numbers of centres due to the presence of Fty-1 initiators in mixtures with wild type cells at different population densities. The upper curve was obtained for 1000 Fty-1 cells and the lower for 500 Fty-1 when mixed with 10,000 wild type. Two separate experiments were performed for each of the mixtures.

To return to the question raised at the beginning of this section, the foregoing indicates that almost as many centres are evoked by Fruity initiators in a test system composed of wild-type responder cells as in the presence of mutant responders. Thus, in the former system the ratio of mutant cells to centres evoked by mutant initiators was about 1 : 60 and in the latter system, 1 : 24. In contrast, the corresponding value for the wild type initiator/responder system is 1 : 2200. It seems therefore that most of the enhanced centre producing capacity of the mutant can be ascribed to an increase in the proportion of initiator cells. Since, however, there is a significant difference (60 against 24) in the ratios obtained with the two test systems, at least a part of this capacity must involve enhanced sensitivity of the mutant responding cells to the initiative stimulus.

*Microscopic observations of Fty-*1 *aggregation*

A population of wild type myxamoebae in the midst of aggregation is truly a remarkable sight. The process begins with greatly increased pseudopodial activity and sudden and drastic elongation of the cells. Active directed motion follows and, as the cells approach each other all travelling in roughly radial paths, the first dim outlines of the cell streams become apparent. With subsequent crowding the streams are clearly delineated and are fortified by the arrival of reinforcements. Major and minor branching occur at the distal ends. A sticky material appears to hold the streams together (Dr B. M. Shaffer, personal communication). The cells move toward the centre which rapidly emerges as a cone-shaped mass. At its peak, the aggregation figure may extend over a diameter of a few millimetres. By the time the last cells are nearing the centre, the aggregate has already taken on the distinctive outlines of the pseudoplasmodium. Depending on the population density, a few hundreds to many thousands of cells may be incorporated in a single aggregate. Photomicrographs of typical wild type aggregations have been published elsewhere (Raper, 1951; Raper & Fennell, 1952).

In view of the drastic decrease in the numbers of cells normally entering the Fruity aggregates, it was of interest to examine the form that the mutant aggregate takes and the way in which the cells enter it. Plates 1 and 2 show a series of time-lapse still photomicrographs of Fty-1 myxamoebae during aggregation. The cells had been dispensed on minimal agar at a population density slightly greater than the optimal. A field was chosen at random and the Petri dish was taped rigidly to the microscope so that serial photographs could be taken. A ×10 ocular and a ×10 objective were used. The first photograph (fig. 1) was taken after 10 hr. of incubation, and the succeeding ones at 13, 13·5, 15·5 and 24 hr. The times respectively involved should not be considered critically since the experiment was performed during a Chicago summer at ambient temperature very close to the upper allowable limit. Thus the time between the beginning of aggregation and fruiting body formation was greatly in excess of the norm. In all other respects the pattern was typical.

A number of features may be noted: (i) there was no evidence of cell elongation or stream formation; (ii) the aggregate remained amorphous even after a considerable number of cells had arrived at the centre and even the completed aggregate had not yet achieved the shape of a typical pseudoplasmodium; (iii) the cells appear to have been in constant random motion prior to their aggregation. It is impossible to follow the movement of more than a few cells even through two photographs.

Estimation of the number of cells which entered this aggregate can only be approximate. It is based on the number of cells in the field and the relative size of the aggregate; from this we estimate that less than 100 cells took part. Notice that in Pl. 2, fig. 5, the field was shifted slightly to include a second and smaller aggregate; probably less than 20 cells entered this one.

Of primary interest is the question of whether one might not be able to identify the initiator cell in a beginning aggregate, by some difference in morphology, motility or other property. The mutant aggregations would seem to be ideal material for a study of this kind because of the small number of cells involved; this is under investigation.

The comparative anatomy of the mutant fruits

Because of the small numbers of cells involved, it was of interest to examine the structure of the mutant fruiting body. Pls. 3 and 4 are photomicrographs of three examples. They were obtained by choosing a fruit whose axis was not perpendicular to the agar and allowing a drop of immersion oil to fall upon it. The oil served to press the fruit down upon the agar and to squeeze excess water from it and clarify the view. The photographs were taken with a bellows-equipped camera over a × 10 ocular and × 90 oil-immersion objective. Pl. 3, fig. 6, is a montage since the fruit occupied two fields. The structure is relatively large and probably came from an aggregate of the same size as that shown in the previous figure. Microscopic examination at several focal planes revealed that the fruit contained about fifty spores, 4 to 6 stalk cells and probably 1 basal disk cell. Pl. 4, figs. 7 and 8 shows views in two focal planes of a smaller fruit containing 9 spores, 2 stalk cells, and probably 1 basal disk cell. Pl. 4, fig. 9 is of interest because of the sharp delineation of the stalk cells.

The results of morphological examination of these and a few other mutant fruits are outlined below. Where possible, comparisons have been drawn with the corresponding anatomical features of the wild type.

Spores. The mutant spores appear indistinguishable from those of the wild type with respect to size, shape and cell-wall thickness. Measurement of the major and minor diameters of 30 mutant spores yielded averages of 7·5 and 4·25μ., or a mean of 5·9μ. Corresponding dimensions for the wild type were 7·1 and 4·5μ., or a mean of 5·8μ. Bonner & Frascella (1949), after a much more extensive study, found a mean of 6·0μ. for the wild type.

Stalk cells. As in the wild type (Raper, 1941; Raper & Fennell, 1952), the mutant stalk cells are firm, thick walled and completely vacuolated. The latter property was made obvious in our examination by the presence of dancing bodies. As seen in these figures, the cells are singly tiered and vertically oriented. The dimensions of four clearly delineated cells ranged between 2·6 and 3·8μ. in width and 28 and 32μ. in length. Raper (1941) noted tiny wild type fruits formed at low population density whose stalk cells were similarly shaped with dimensions of 3·5 × 15μ. Raper's findings also indicated great plasticity of stalk cell shape since in slightly thicker stalks the cells became progressively more isodiametric.

At the other extreme are the stalks of relatively huge wild type fruits formed on growth plates at very high population density. These contain tiers of 10–15 cells all isodiametric (Raper, 1941). The same can be said of the mutant fruits formed under similar circumstances although none have been seen that are that thick.

In the wild type, the stalk cells are encased in a tubular tapered cellulose sheath (Raper & Fennell, 1952). Sheaths were also observed by us in the mutant, although the nature of the material was not determined. In the tiny fruits of both varieties, the sheaths are not readily distinguishable from the outer cell walls.

Gross proportions of the fruits. Table 4 lists the lengths of six mutant fruits and the ratio of cells present to fruit length; the shortest was 45μ. and the longest 190μ. The mutant fruits on growth plates commonly attain a height of 1 or 2 mm. In contrast, of the wild type fruits examined by Raper (1941), the smallest was 750μ. and the largest 4500μ. The ratio values in Table 4 indicate a constancy of gross proportions regardless of the number of cells present; this point requires more extensive study. Similar data for the wild type are not available. The ratio of the diameter of the spore mass to the length of the stalk was determined for fruits of very different sizes by Raper (1941), who found a constant ratio of 0·1; corresponding measurement of a limited number of mutant fruits yielded a constant value of 0·2.

Table 4. *The relation between the lengths of the mutant fruits and the numbers of cells comprising them*

Fruit	No. of cells	Fruit length (μ.)	Ratio, cells : length
1	12	70	0·17
2	13	45	0·29
3	15	66	0·23
4	19	99	0·19
5	32	125	0·25
6	56	190	0·29

Ratio of spores and stalk cells. On the basis of limited data, the ratio of stalk cells to spores in the mutant was found to be relatively constant despite differences in absolute numbers and to range between 0·1 and 0·2. Bonner & Slifkin (1949), in an extensive study of the parental strain under slightly different environmental conditions, found a ratio of 0·14.

From the foregoing, it would appear, that aside from considerations of absolute size, the mutant fruits do not differ from those of the wild type to any significant extent. This implies that the processes which in the parental variety determine the morphology of the fruit, both macro-and microscopic, proceed normally within the mutant unaffected by the genetic alteration. The capacity of these organisms for developmental regulation (i.e. constancy of morphogenetic properties regardless of the absolute size of the cell assembly) should be noted. From fruits measured in millimetres and containing many thousands of cells to fruits measured in microns and containing relatively few cells, the gross and microscopic aspects are preserved intact.

The regulatory properties of the fruiting process

As shown by Raper (1941) and by Bonner & Slifkin (1949), the fruit of *Dictyostelium discoideum* maintains a constancy of gross form, proportions and histological features whether it be composed of hundreds of cells or of many thousands. Now the fruity mutant has extended this regulatory scale downward to include structures composed of as few as 12 cells and perhaps less. One may ask the question as to what in a developing population of myxamoebae determines whether a given individual is to become a spore, stalk, or basal disk cell and further, what determines the proportions of the types within the differentiated assembly. A partial answer has been gained through studies (Raper, 1940; Bonner, 1944) which indicate that the fate of a cell in these regards is governed by its relative position in the aggregate and pseudoplasmodium. Experimentally contrived changes of position result in predictable alterations of that fate. This suggests that the differentiation of the myxamoebae is mediated by cell/cell interactions and that the proportions of the various cell types represent resultants among the matrix of interactions. Study of synergistic development by paired combinations of morphogenetically-deficient mutant strains (Sussman, 1954) has provided strong support for this concept and the additional information (Sussman & Lee, 1955) that in those synergistic relations examined, the material exchanged between the partners is not diffusible but rather is passed by direct cell contact. The pertinence of the Fruity mutant to the problem lies in the small numbers of cells that take part in the organization. If 9 pre-spore cells can regulate the transformation of 2 pre-stalk cells or vice versa, then the interactions attending the transformations must be extremely powerful in the sense that they need not be actions to which many cells contribute. Rather must it be that the control proceeds through direct effects of one or at most a few cells upon another, with perhaps the additional requirement of correct geometric orientation of effector and effectee. It would seem that the Fruity mutant may provide the means for a delicate attack upon this problem.

In addition, it has been observed that extracts or washings of the Fruity myxamoebae, when incorporated into the minimal agar, induce the wild type to form phenocopies of the mutant aggregations and fruits. Our best preparations have provided more than a tenfold increase in the expected number of aggregative centres. This increase does not result from growth of the wild type cells on the plates or the presence of viable mutant cells in the extracts or washings. The enhanced aggregative capacity is not maintained by progeny of spores taken from the experimental plates. The chemical and biological features of this phenomenon are currently being studied.

The dwarf mutant of Dictyostelium discoideum

Some time after the investigation of Fty-1 had been started, it was recalled that a mutant Dwarf Dw-1, isolated 2 years previously, resembled the Fruity mutant in many respects. At the time of its isolation the small size of the fruits of Dw-1 rather than the increase in their number held our attention since we then possessed no preconceptions regarding genetically determined increases in the number of initiator cells. Re-examination of Dw-1 has revealed that it too is a 'fruity' stock although not as extreme in this respect as Fty-1. A study of aggregative performance on minimal agar revealed that the response of Dw-1 to population density changes was about the same as that of Fty-1, but that the ratio of centres to cells of the former is 1 : 40 as against 1 : 24 for the latter. The fact that the two mutants show slightly different characteristics indicates that initiative capacity in *Dictyostelium discoideum* is controlled by a multiplicity of genetic determinants.

Aggregation of the Bushy mutant of Dictyostelium discoideum

Another mutant designated NC-4 bu, was isolated some time ago and its overall morphogenetic pattern described (Sussman & Sussman, 1953). The myxamoebae of NC-4 bu appear to aggregate in normal fashion. The mutant aggregate is not, however, transformed into the usual pseudoplasmodium, but instead remains stationary and upright and becomes heavily papillated. From each papilla emerges a tiny fruiting body with spore mass and stalk. An analysis of the aggregation of this mutant was performed by the procedure previously described. Figure 5 shows the relation between centre formation and population density. Curve I was obtained with populations of 2 × 10^4 cells, curve II with 1 × 10^4 cells, and curve III with 5 × 10^3. As with the wild type, no centres were formed by the Bushy cells at very low population densities (*c*. 70 cells/mm.^{2}); at very high densities the number of centres was depressed by interactions between the closely apposed potential centre-forming agencies. At an intermediate density a maximal number of centres was produced, proportional to the number of cells present. Table 5 is a numerical summary of these results. The mean ratio of centres : cells for the Bushy strain was about 1 : 1800, to be compared with the 1 : 2200 ratio for the wild type. In view of

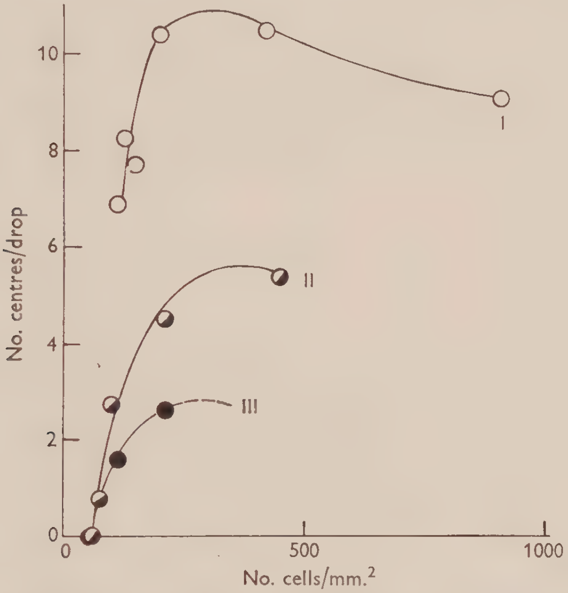

Fig. 5. The relation between centre formation and population density for the mutant, Bu-1. Curve I was obtained with aliquots of 20,000 cells, ○; curve II with 10,000, ◑ ; and curve III, with 5000 ●.

Table 5. *Centre formation by the Bushy mutant at optimal population density*

No. of cells	No. of centres	Ratio
20,000	10·9	1 : 1830
10,000	5·5	1 : 1820
5,000	2·8	1 : 1780
	Mean	1 : 1810

the random sampling error inherent in this technique, the difference may not be significant. However, the optimal population density for Bushy (about 350 cells/mm.^{2}) does differ significantly from the corresponding value for the wild type (200 cells/mm.^{2}). In addition, a difference was observed in the rates of development. All wild type aggregates appear well before 24 hr. of incubation on washed agar. Aggregates of Bushy cells continued to appear until 48 hr. of incubation. At high population densities single Bushy aggregates produced multiple fruiting structures, as many as four or five fruits arising therefrom. At low densities where each aggregate contained fewer cells only single fruits appeared.

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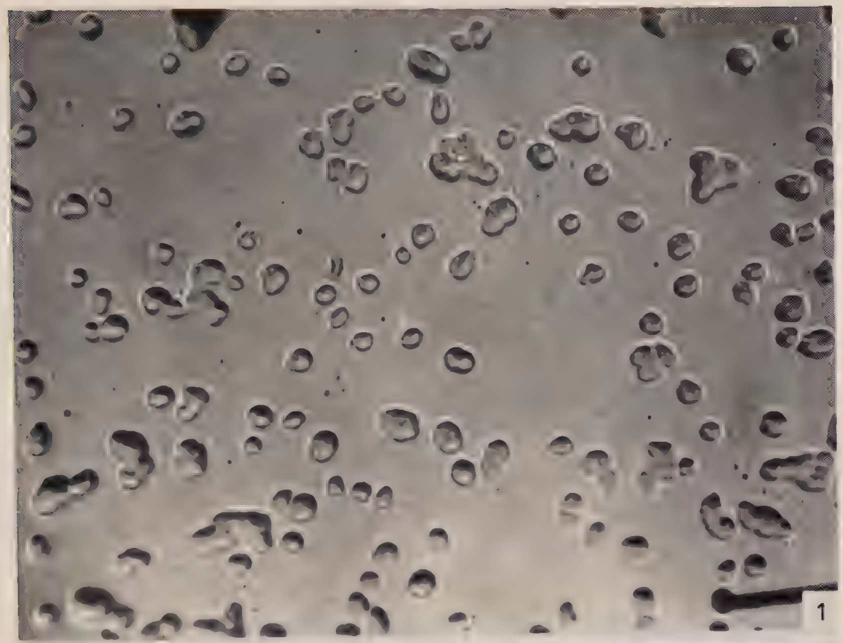
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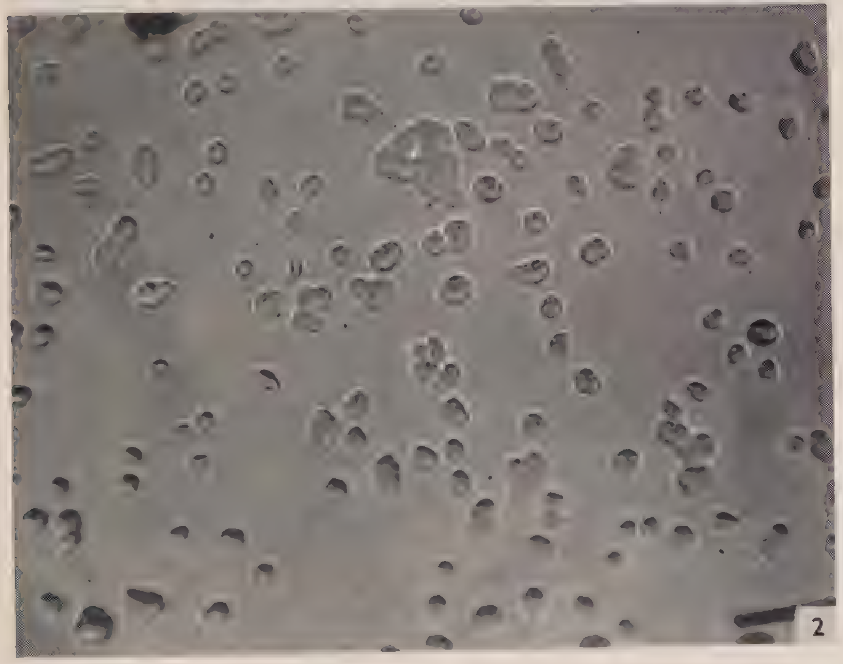
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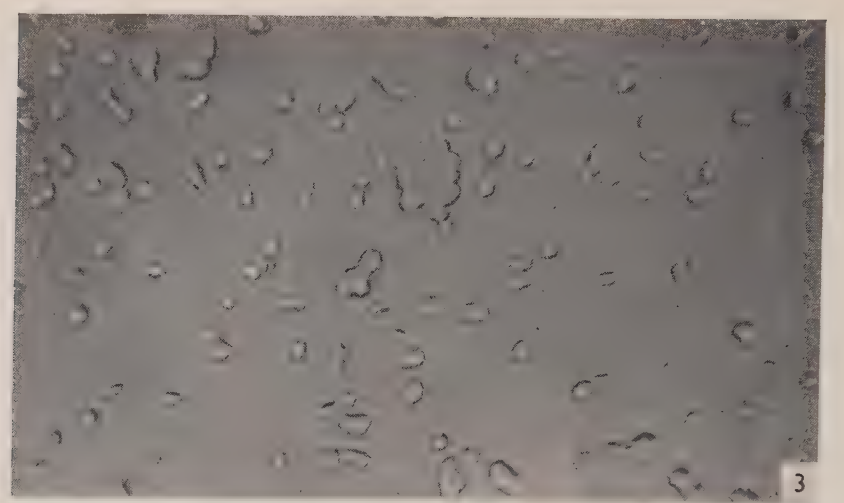
M. SUSSMAN—MUTANTS OF *DICTYOSTELIUM DISCOIDEUM*. PLATE 1



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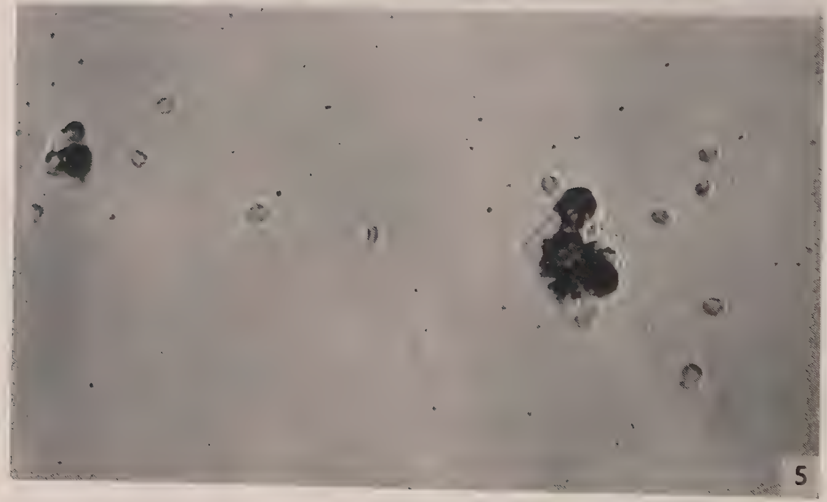

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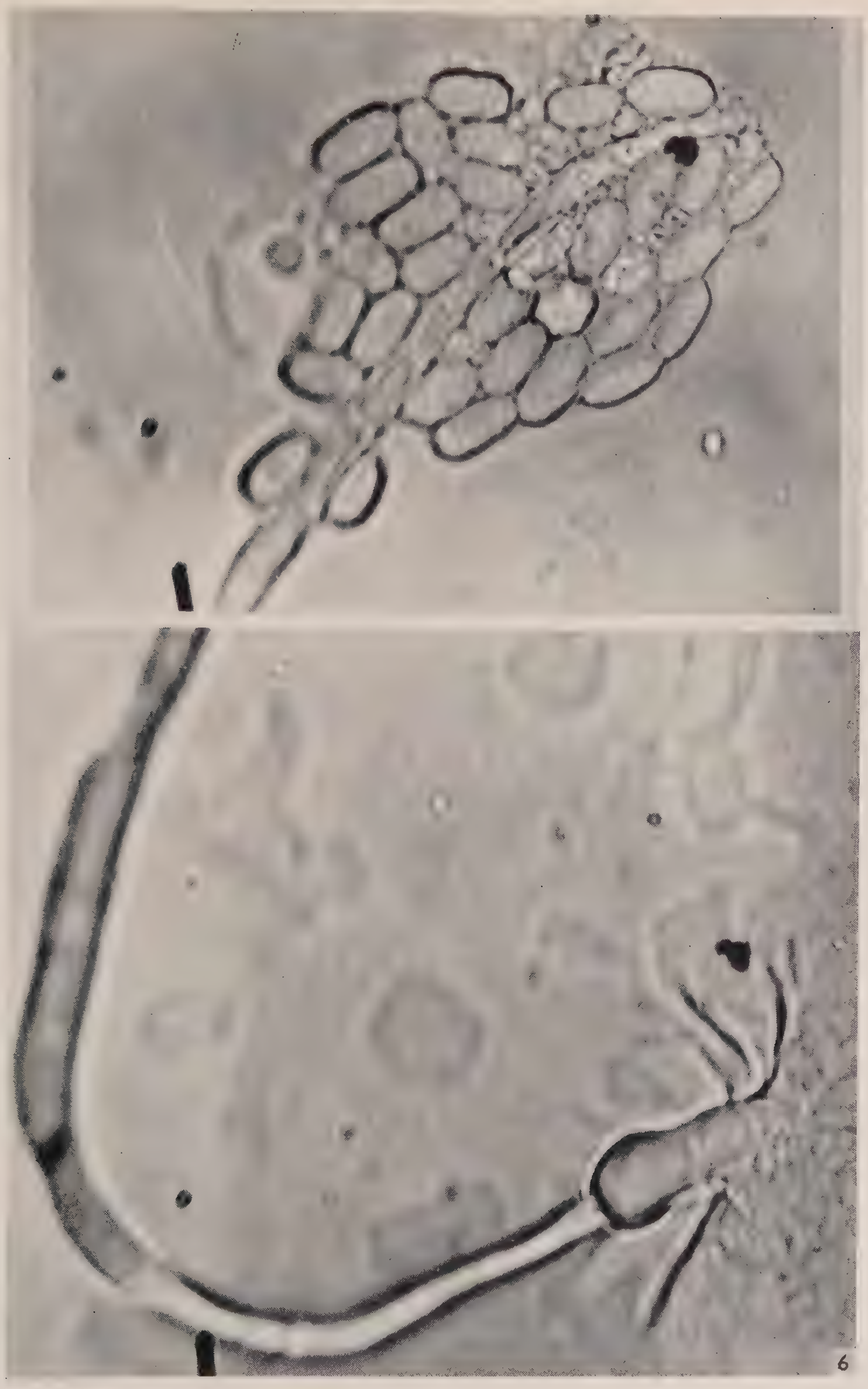
M. SUSSMAN MUTANTS OF *DICTYOSTELIUM DISCOIDEUM*. PLATE 2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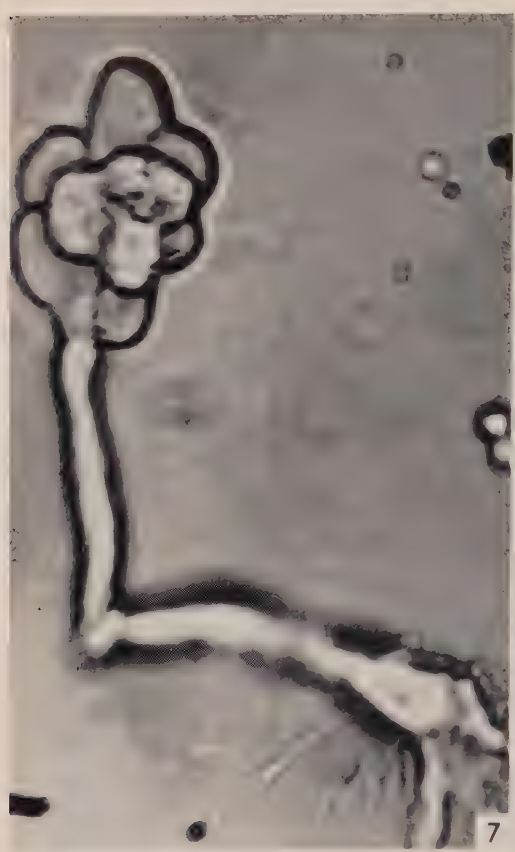
M. SUSSMAN—MUTANTS OF *DICTYOSTELIUM DISCOIDEUM*. PLATE 3



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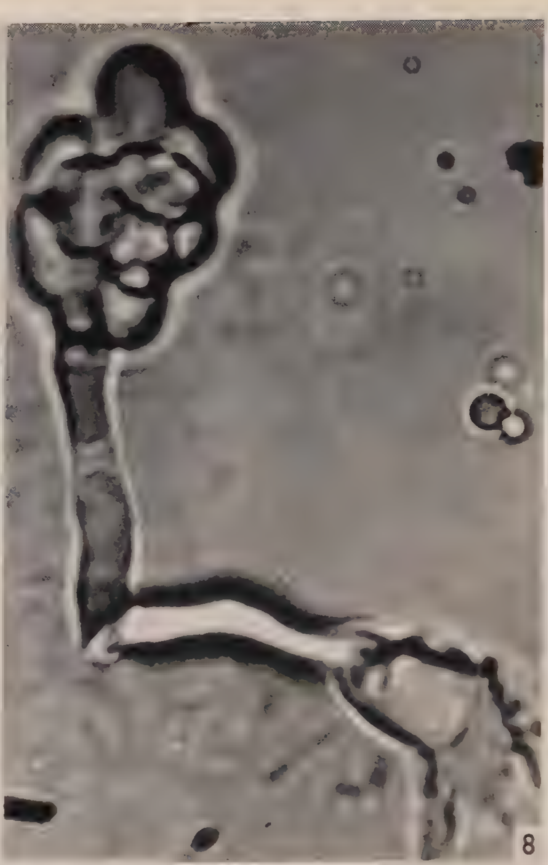

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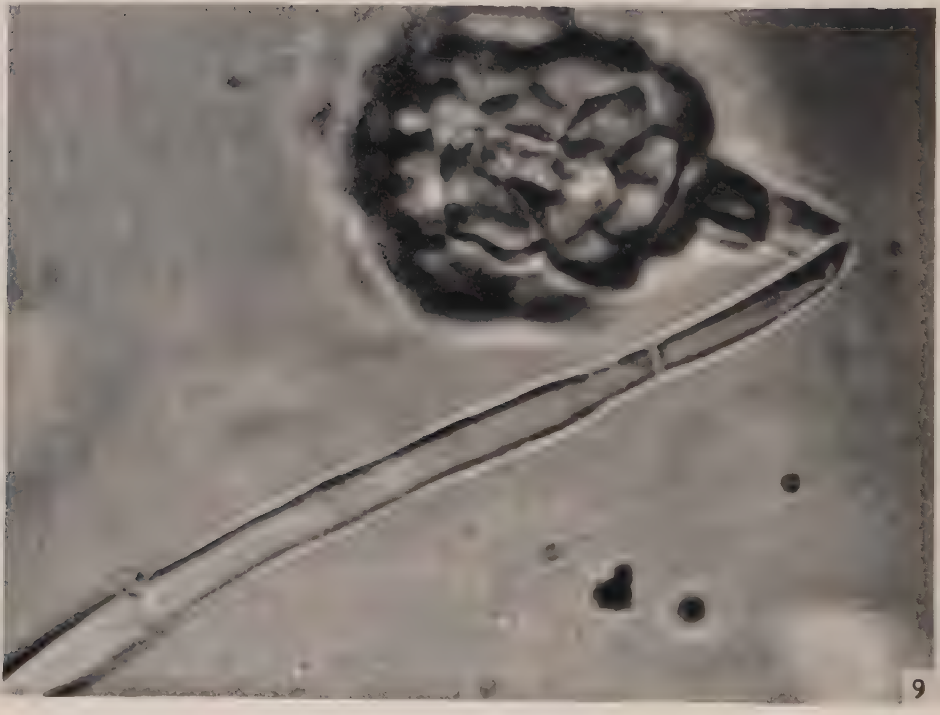

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M. SUSSMAN MUTANTS OF *DICTYOSTELIUM DISCOIDEUM*. PLATE 4

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EXPLANATION OF PLATES

Plate 1

Figs. 1, 2. Serial photomicrographs of an Fty-1 aggregation. Fig. 1 (upper) was taken after 10 hr. of incubation on minimal agar; the lower photograph (fig. 2) after 13 hr. Magnification, ×300.

Plate 2

Figs. 3–5. Serial photomicrographs of an Fty-1 aggregation. Fig. 3 (upper) was taken after 13·5 hr. incubation on minimal agar; fig. 4 (middle) after 15·5 hr.; fig. 5 (lower) after 24 hr. Magnification ×300.

Plate 3

Fig. 6. A photomicrograph of a mutant fruit *in situ*. This is a montage of two fields. Magnification ×1650.

Plate 4

Figs. 7–9. Figs. 7, 8 (upper left and right). Photomicrographs in two focal planes of a mutant fruit *in situ*. Magnification ×1760. Fig. 9. Photomicrograph showing a stalk cell of a mutant fruit. Magnification ×1760.

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The Electron-dense Bodies of *Mycobacterium phlei*

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SUMMARY: A method is described in which identical groups of micro-organisms are viewed first in the light microscope and then in the electron microscope. A direct comparison was made between electron micrographs and preparations stained with specific metachromatic, chromatinic and mitochondrial stains. In a study of the electron-opaque granules of the mycobacteria it was shown that they contain metaphosphates and show metachromasia. They are situated between the chromatinic bodies and cannot be identified with mitochondria.

There is still considerable controversy concerning the nature of the electron-opaque granules which are found in the mycobacteria and other micro-organisms. It was realized that more specific information concerning these structures could be obtained by a comparison of identical organisms viewed under different experimental conditions. By mounting the bacteria on electron microscope grids it was possible to photograph groups of organisms in the light microscope and then locate the same groups in the electron microscope. In this way stained preparations and electron micrographs were compared directly, and preparations stained by different procedures were compared among themselves. This method has provided useful evidence concerning the dense bodies of the mycobacteria and has also thrown light on the problem of the bacterial nucleus.

METHODS

The strain of *Mycobacterium phlei* (Söhngen) used in these experiments was obtained from the National Collection of Type Cultures in 1943 and has been subcultured subsequently at the Strangeways Laboratory. It is particularly suitable for a study of internal structure owing to its large size, ease of growth and formation of abundant dense bodies under normal cultural conditions. Throughout the present experiments the bacteria were grown on plates consisting of equal parts of glycerol agar and embryo extract.

The comparative microscope methods were carried out with the bacilli mounted on 'formvar' films covering electron microscope grids. Films strong enough to withstand the various staining processes were formed from a 0·8% solution of formvar in ethylene dichloride cast on water. There was a tendency for the formvar films to float off the grids in some of the solutions, but this was prevented by coating the grids with a thin layer of polybutene before picking up the formvar films. When a grid is dipped in a 1·0% solution of polybutene in xylene and placed on filter-paper to dry, a thin sticky layer forms over the grid bars without obscuring the holes (Drummond, 1950). Flat copper grids (Smethurst High-Light Ltd.) were used to give an even field for light microscopy.

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To mount the organisms on a grid a square of the inoculated glycerol agar + embryo extract plate was cut out and placed on a glass slide in a Petri dish. A print was taken on the grid by inverting it, with the formvar film downwards, on the growth of organisms on the surface of the plate; when the grid was removed a proportion of the organisms remained attached to the formvar film. When the plate was rather soft some of the medium also stuck to the grid but was easily removed by gentle washing in distilled water. The organisms were fixed by placing the grid face upwards on several layers of filter-paper soaked in 5·0% (v/v) formalin for 30 min. Sufficient fixative diffused through the holes in the formvar film. After fixation the grids were washed in distilled water and placed on filter-paper to dry. The organisms could then be viewed in the electron microscope, with or without previous metal shadowing.

When the organisms were stained, the grid was treated like a coverslip preparation and carried through the various staining, washing and differentiating solutions. Specific metachromatic, chromatinic and mitochondrial stains were used.

To make a direct comparison of light- and electron-microscope pictures of identical organisms, the stained grid was mounted in cedar wood oil between a thin slide and coverslip so that the high-power oil-immersion objective of a light microscope could be used. The central four squares of the copper grids were marked and were easily found. Groups of stained organisms lying in these or adjacent squares were photographed on 35 mm. film and enlargements made from the negatives. The grid was then soaked in xylol to remove the cedarwood oil, and after drying was ready for examination in the electron microscope. The central squares were again viewed, and with the help of photographs, already prepared, the same groups of organisms were found with little difficulty and photographed again with the electron microscope. The electron micrographs were taken on a Siemen's electron microscope in the Cavendish Laboratory, Cambridge, at a primary magnification of *c.* × 8000.

A similar procedure was followed when comparing results with two different stains. After being stained for the first time and photographed, the organisms on the grid were decolorized and restained by the second procedure. Again the same group of organisms was easily found and re-photographed.

RESULTS

Metachromatic stains

Bacteria on grids were stained by the standard procedure with Loeffler's methylene blue. In the light microscope a normal 24 hr. culture was seen to consist of groups of rods and long filaments in the stage of filamentous proliferation (Brieger, Cosslett & Glauert, 1954). The organisms were stained blue and had bright red granular inclusions. These red metachromatic granules varied considerably in size, and some of them had an admixture of blue colour so that they appeared red-purple or black.

In the electron microscope the organisms were seen to contain well-defined electron-opaque bodies which corresponded with the metachromatic granules in number and position. When identical groups of organisms were viewed, first in the light microscope and then in the electron microscope, it was found that the red granules and the electron-opaque bodies occupied exactly the same positions (Pl. 1, fig. 1*a*, *b*). In the light microscope photograph the red granules have been marked with arrows.

Confirmatory evidence for the metachromatic nature of the dense bodies was given by Neisser's stain for volutin. Again there was a direct correlation between the volutin granules of the stained preparations and the electron-dense bodies (Pl. 1, fig. 2*a*, *b*). The granules were not so clearly seen as with methylene-blue staining because of the greater density of the cytoplasmic staining which masked other structures. The granules failed to stain metachromatically with toluidine blue.

May-Grünwald and Giemsa stains

In preparations of the mycobacteria stained with the May-Grünwald stain alone a striking arrangement of red granules was seen within the pale blue rods and filaments. The red bodies could be identified with the electron-opaque granules by a comparison of light and electron-microscope pictures (Pl. 1, fig. 3*a*, *b*). The vacuolated appearance of the cytoplasm in the electron micrograph is typical of these bacteria at certain stages of development, and in this organism the effect was enhanced by electron bombardment.

With the complete May-Grünwald and Giemsa stain the red granules were still present in the rods and filaments. In some parts of the preparations they were clearly seen (Pl. 1, fig. 4*a*), but in other areas the background staining of the cytoplasm was so intense that the red bodies and other internal structures were barely visible. They could be made more evident by the use of appropriate filters or by allowing the overlying stain to fade. In the electron microscope most of the organisms appeared very dense (Pl. 1, fig. 4*b*), but they could be made more transparent by electron bombardment. After this treatment the electron-opaque granules were once more apparent, and comparison with the corresponding light-microscope photographs confirmed that they occupied the same positions as the red granules (Pl. 1, fig. 4*a*, *c*).

Chromatinic stains

The organisms on an electron-microscope grid were stained by the HCl-Giemsa method (Piekarski, 1937; Brieger & Robinow, 1947), and the familiar picture of rows of irregularly shaped chromatinic bodies was obtained. In the electron microscope the organisms appeared very dense owing to the Giemsa stain, and the electron-opaque granules were either extracted or decreased in density by the hydrolysis procedure. A comparison of light and electron microscope photographs (Pl. 1, fig. 5*a*, *b*) indicated some parallel between the chromatinic structures and the denser areas in the electron micrographs, but it can be assumed that at least part of the density was due to the deposition of stain at these sites.

It was noticed that in preparations stained with methylene blue, the blue structures which lay between the red granules resembled the chromatinic bodies of the HCl-Giemsa stain. A direct comparison of identical groups of organisms stained by the two methods showed that the 'blue' structures and the chromatinic bodies were exactly the same in shape and position and that the metachromatic granules were no longer visible in the HCl-Giemsa preparations (Pl. 2, fig. 6*a*, *b*). The granules which had stained red-purple or black with methylene blue were still present after hydrolysis and presumably only the red-staining substance had been removed from them. One of these granules can be seen in Pl. 2, fig. 6*a*, and is marked with a double arrow.

Mitochondrial stains

The location of oxidative-reductive sites in the mycobacteria was studied with triphenyltetrazolium chloride (TTC) and Janus Green B. After 24 hr. of growth on the surface of a glycerol agar + embryo extract plate, prints were taken on formvar-covered electron microscope grids in the usual way. These grids were then placed face downwards on several layers of filter-paper soaked in a glucose + phosphate medium containing 0·01% (w/v) TTC or 0·001% (w/v) Janus Green B. The preparations were incubated at 37° and grids removed at various times, mounted without fixation in a drop of glucose + phosphate medium and observed in the light microscope.

After $1\frac{1}{2}$ hr. incubation coloured granules were observed in some of the TTC preparations and were presumed to represent deposits of the red formazan formed by reduction of TTC. The colour was faint and the granules could only be observed and photographed with the diaphragm of the condenser stopped down considerably. As with the other stains, identical groups of organisms were viewed in the electron microscope and a comparison was made (Pl. 2, fig. 7*a*, *b*). In some of the organisms the reduction sites and the electron-opaque granules occupied the same positions but the stained granules were frequently much larger than the dense bodies. Sometimes the deposits of dye were unaccompanied by any corresponding dense granules. No similar stained granules were observed in the mycobacteria grown in contact with Janus Green B even after several hours of incubation. By careful observation a few small peripheral particles were seen but they did not correspond in number or position to the electron-opaque bodies.

Results of treatment with ribonuclease and deoxyribonuclease

Samples of ribonuclease and deoxyribonuclease (obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio, U.S.A.) were made up as 0·1% (w/v) solutions in glass-distilled water. The deoxyribonuclease solution was further diluted with an equal volume of 0·15 M-$MgSO_4$ before use. Treatment of the organisms on an electron-microscope grid with either ribonuclease or deoxyribonuclease for 1 hr. at 37° failed to remove the electron-opaque granules from most of the organisms (Pl. 2, figs. 8, 9). Similarly, the metachromatic granules and the red bodies of the May-Grünwald preparations were unaffected. In one group of organisms the dense granules were differentiated into a darker cortex and lighter core but this was considered to be the effect of electron bombardment and not of the action of ribonuclease. Treatment with ribonuclease followed by deoxyribonuclease also failed to remove the granules.

Treatment with perchloric acid and trichloroacetic acid

Extraction of the organisms with cold 10% perchloric acid (a 60%, w/w, solution diluted with distilled water) for 24 hr. caused the disappearance of many of the dense bodies from the electron micrographs (Pl. 2, fig. 10), but in a few organisms a resistant cortex of dense material remained. A similar extraction with cold 10% (w/v) trichloroacetic acid also removed the dense bodies (Pl. 2, fig. 11). There was parallel disappearance of metachromatic and red granules from the stained preparations.

DISCUSSION

Earlier observations on the internal structures of mycobacteria viewed in the electron microscope were summarized by Brieger, *et al.* (1954). The location and development of the electron-opaque granules during reproduction were studied, and it was concluded that they were not the nuclei. The results obtained with the method described in the present paper indicate more specifically the nature of the dense bodies. Observations with methylene blue and the Neisser stain identify these electron-dense bodies with the metachromatic volutin granules revealed by these techniques. Similar results were obtained by Winkler (1953) with a bacillus, and by Smith, Wilkinson & Duguid (1954) with *Aerobacter aerogenes*. It was concluded by these authors that the electron opaqueness and staining characteristics of the dense bodies is due to their content of metaphosphates. A similar interpretation for the dense bodies of the mycobacteria is supported by the work of Winder & Denneny (1954), who found a direct correlation between the amount of metaphosphate present and the number and size of the metachromatic granules in *Mycobacterium smegmatis* and in *M. tuberculosis*. The extraction of the granules by perchloric acid and trichloroacetic acid also supports this interpretation as suggested by Grula & Hartsell (1954) for *Caulobacter vibrioides*.

Although it seems certain that the most striking properties of the electron-dense bodies of the mycobacteria are due to their metaphosphate content they also contain other substances. The quantity of metaphosphate present in the cells is not sufficient to account for the total volume of the dense bodies (Smith, *et al.* 1954), and it seems likely that they also contain ribonucleoprotein, organic material and other substances. Winder & Denneny (1954) noted that it was difficult to extract the metaphosphate separately from the ribonucleic acid, and suggest that they are bound in a similar manner. However, the fact that the density and staining characteristics of the granules was unaffected by ribonuclease indicates that they did not contain more than small amounts of ribonucleoprotein. When the granules are extracted by acids or volatilized in the electron beam they frequently differentiate into a darker cortex and lighter core, which suggests that a residue of organic material remains.

In one of the earliest interpretations of the electron-dense bodies of the mycobacteria it was suggested that they are nuclei (Knaysi, Hillier & Fabricant, 1950; Knaysi, 1951). However, this interpretation has always seemed doubtful to the present authors because of the behaviour of the dense bodies during reproduction. As noticed by other workers also (Winkler, 1953; Takeya, Koike, Uchida, Inoue & Nomiyama, 1954), the dense bodies do not appear to be essential for the development of the organisms and groups of cells containing no granules reproduce in the normal way. The results described in the present paper with the HCl-Giemsa stain prove that the chromatinic bodies of the mycobacteria examined are not identical with the dense bodies and lie between them (Pl. 2, fig. 6*a*, *b*). A similar result was reported by Tronnier (1953) for *Corynebacterium diphtheriae*.

Observations with mitochondrial stains show how misleading these procedures can be when applied to bacteria. Some parallel can be drawn between reduction sites and the positions of the electron-opaque granules, but in general the deposits of dye are far larger than the dense bodies (Bielig, Kausche & Haardick, 1952). The impression is gained that the dense bodies merely act as centres around which the reduced dye is deposited. This interpretation is supported by the fact that in some areas dense bodies are seen with no accompanying stained granules. It was observed by Weibull (1953) that the red formazan resulting from the reduction of triphenyltetrazolium chloride in *Bacillus megaterium* first appeared as a number of scattered particles and then coalesced to form the familiar granular deposits; it is possible that a similar process occurs in the mycobacteria used here. As long ago as 1951 Wallhäusser reported the movement of reduction sites within living cells; these observations throw grave doubts on the suggestion that the electron-dense bodies represent the mitochondria of bacterial cells (Mudd, 1953).

It was hoped that the application of the May-Grünwald and Giemsa stain to the mycobacteria would provide the same clear differentiation of ribonucleoprotein and deoxyribonucleoprotein as obtained in mammalian cells (Jacobson & Webb, 1952), in which the ribonucleoprotein stains blue and the deoxyribonucleoprotein red. However, the metachromatic volutin granules of the mycobacteria also stained red with the May-Grünwald stain because of their metaphosphate content (Hartman & Payne, 1954). The fact that the red colour of the granules was not due to their content of deoxyribonucleoprotein was verified by the application of deoxyribonuclease which failed to remove them. The problem of the location of the bacterial nucleus remains to be solved, but the results of this paper show that the electron-opaque granules of the mycobacteria used here are not the nuclei, even though they may be related to the "nuclear apparatus".

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EXPLANATION OF PLATES

Micro-cultures of *Mycobacterium phlei* (Söhngen) grown on glycerol agar + embryo extract plates; incubation at 37·5°.

Plate 1

Comparative light- and electron-microscope photographs of impression prints taken on electron microscope grids.

Fig. 1*a*. 24 hr. growth stained with Loeffler's methylene blue. The rods and filaments are blue and contain bright red, metachromatic granules (arrows). Light microscope; × 2900.

Fig. 1*b*. Electron micrograph of the same filament as in Fig. 1*a*. The electron-opaque granules correspond with the metachromatic bodies. Electron microscope; × 7200.

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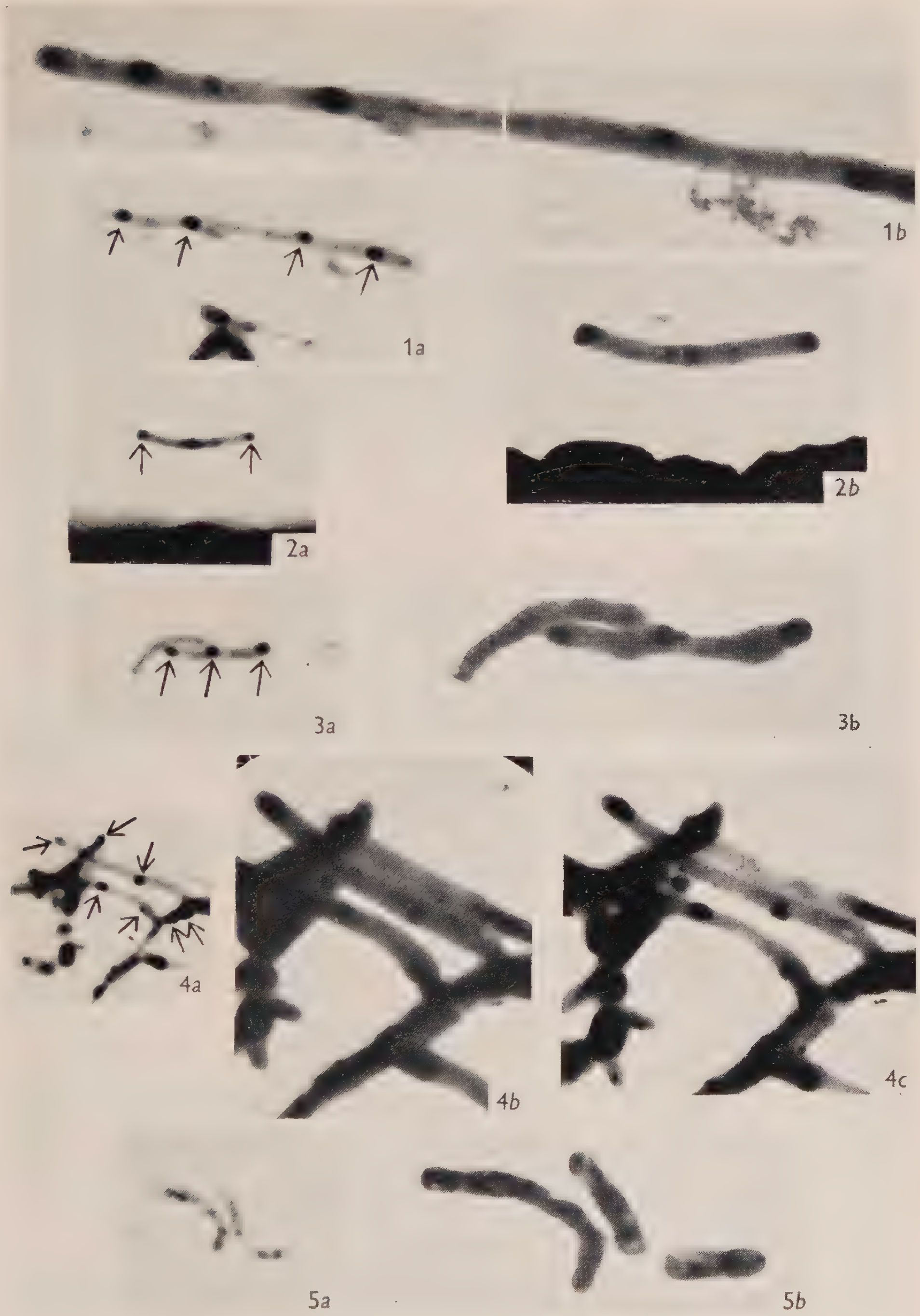
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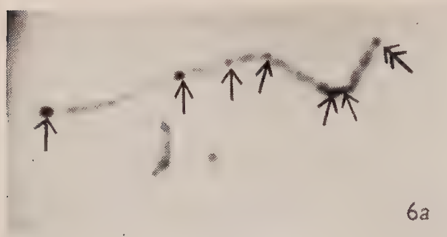
A. M. Glauert & E. M. Brieger—Electron-dense bodies in *M. phlei*. Plate 1



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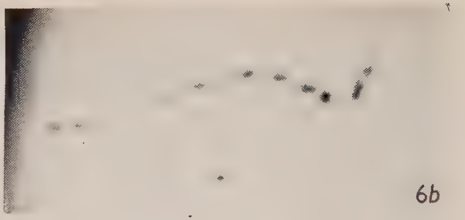

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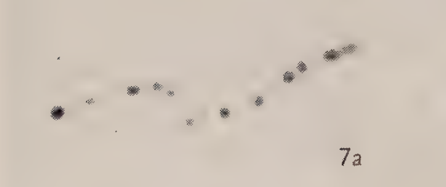

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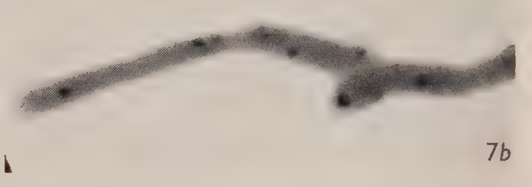

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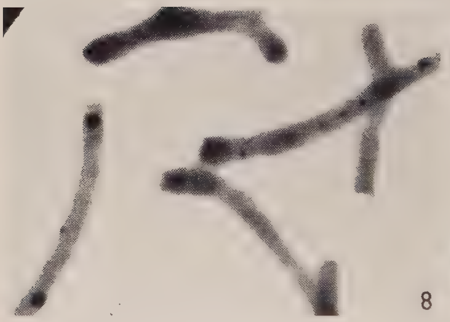

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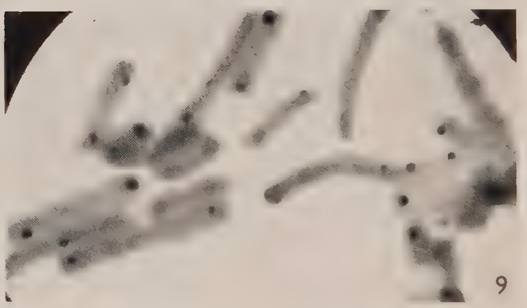

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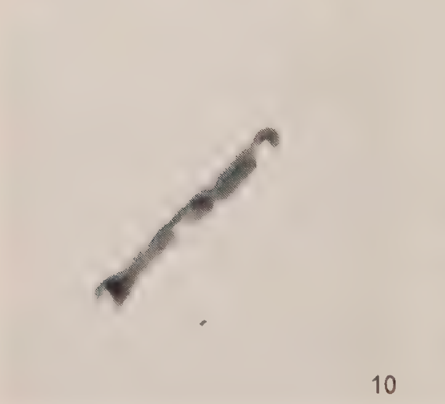

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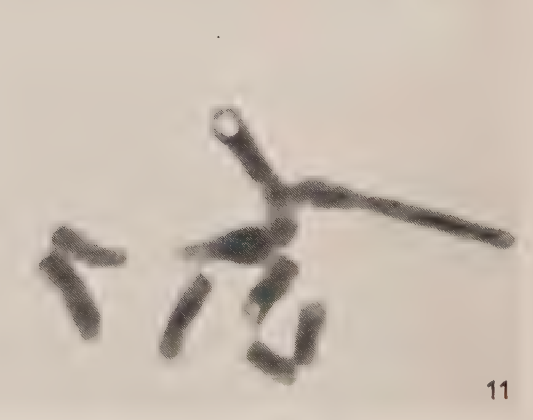

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A. M. Glauert & E. M. Brieger—Electron-dense bodies in *M. phlei*. Plate 2

Fig. 2*a*. 2 hr. growth stained with Neisser's stain. The volutin granules are marked with arrows. Light microscope; ×3100.

Fig. 2*b*. Electron micrograph of the same organism as in Fig. 2*a*. The volutin granules are dense in the electron microscope; ×6600.

Fig. 3*a*. 4 hr. growth stained with the May-Grünwald stain. The organisms are blue and contain red granules (arrows). Light microscope; ×2500.

Fig. 3*b*. Electron micrograph of the same group of organisms as in Fig. 3*a*. The dense bodies correspond with the red granules of the stained preparation. Electron microscope; ×6500.

Fig. 4*a*. 6 hr. growth stained with May-Grünwald and Giemsa stain. A group of branching filaments, stained blue, with red granular inclusions (arrows). Light microscope; ×3400.

Fig. 4*b*. Electron micrograph of the same group of filaments as in Fig. 4*a*. The density of the cytoplasm masks the internal structures. Electron microscope; ×6800.

Fig. 4*c*. Electron micrograph of the same group of filaments as in fig. 4*a* and *b* after electron bombardment in the electron microscope. The cytoplasm is more transparent to electrons and the dense bodies are visible. They correspond to the red granules of the light microscope picture. Electron microscope; ×6800.

Fig. 5*a*. 24 hr. growth stained with HCl-Giemsa. The organisms contain rows of chromatinic bodies. Light microscope; ×2800.

Fig. 5*b*. Electron micrograph of the same organisms as in fig. 5*a*. The Giemsa stain makes the cytoplasm very dense. There is some parallel between the chromatinic bodies of the light microscope picture and the denser parts of this electron micrograph. Electron microscope; ×6500.

PLATE 2

Comparative light- and electron-microscope photographs of impression prints taken on electron microscope grids.

Fig. 6*a*. 24 hr. growth stained with Loeffler's methylene blue. The red metachromatic granules are marked with arrows. One granule, which appeared red-purple, is marked with a double-headed arrow. The other internal structures are stained blue. Light microscope; ×3000.

Fig. 6*b*. The same filament as in fig. 6*a* stained with HCl-Giemsa. The chromatinic bodies correspond exactly with the blue structures of the methylene-blue stain. The metachromatic granules are no longer visible, but the red-purple granule, which had an admixture of blue colour, is still present. Light microscope; ×3000.

Fig. 7*a*. 16 hr. growth and grown for a further 4 hr. on a glucose + phosphate medium containing 0·01 % triphenyltetrazolium chloride. A long filament contains granular deposits of formazan. Light microscope; ×3000.

Fig. 7*b*. Electron micrograph of the same filament as in fig. 7*a*. The filament contains some small electron-opaque granules. There is some correspondence between the positions of these dense granules and the formazan deposits, but the dense bodies are much smaller than the stained granules. Also some of the stained granules have no corresponding dense bodies. Electron microscope; ×7400.

Electron micrographs of *Mycobacterium phlei* after various extraction procedures.

Fig. 8. 4 hr. growth treated with ribonuclease for 1 hr. at 37·5°. The electron-opaque granules are still present. ×7400.

Fig. 9. 28 hr. growth treated with deoxyribonuclease for 1 hr. at 37·5°. The electron-opaque granules are still present. ×7000.

Fig. 10. 4 hr. growth treated with cold perchloric acid. The dense bodies have been extracted. ×8100.

Fig. 11. 28 hr. growth treated with cold trichloroacetic acid. The dense bodies are no longer present. ×7800.

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Cytological Studies of Nuclear Division in *Salmonella typhimurium*

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SUMMARY: Physiological studies recently led to the development of a technique for obtaining synchronous nuclear division in *Salmonella typhimurium*. By using this method we have attempted to identify the morphological changes characteristic of nuclear division. The picture obtained is that of a gradual shift from a round nuclear body via 'wedge'- and 'bar'- to 'dumbbell'-like configurations. Mitotic patterns were not observed in these small cells. An indication was obtained that at an early stage of nuclear division the nucleus has already doubled with respect to at least one of its physiological functions. It is discussed to what extent the present studies by selective staining may be used to identify the state of the nucleus as observed by electron microscopy.

In recent years many cytological observations of the bacterial nucleus have been reported, leading to the putting forward of different theories about the cytology of nuclear division in bacteria (Robinow, 1946; DeLamater, 1951). Until now it has been impossible to test such theories rigorously because cultures in which nuclear division occurs in a random fashion do not permit the identification of the physiological states of the nuclei under observation. With a view to removing this limitation, a system was developed in this laboratory for synchronizing nuclear division in large populations of *Salmonella typhimurium* (Maaløe & Lark, 1954; Lark & Maaløe, 1954). By studying populations of synchronized cells, fixed at various times relative to the beginning and end of nuclear division, it was thought that it might thus be possible to identify a cytological sequence of events for the division of the bacterial nucleus. The present paper presents a cytological study of this system by means of selective staining techniques, as a preliminary to a more detailed study, by means of electron microscopy of serial sections, which is now being carried out by Dr A. Birch-Andersen. Preliminary results of this latter investigation are contained in the next paper (Birch-Andersen, 1955).

METHODS

The properties of the strain of *Salmonella typhimurium*, the liquid and solid media used, and the induction of synchronous nuclear division by means of the single temperature shift method were described previously (Lark & Maaløe, 1954). A broth culture of *S. typhimurium* which had been growing exponentially at 25° for more than ten generations was allowed to reach a density of 10^7 cells/ml. At this time aeration was started to maintain optimal aerobic growth conditions. When the density had increased to about 5×10^7 cells/ml. the temperature of the culture was raised to 37° by the addition of an appropriate volume of broth warmed to 90°. The culture was then grown at 37° with aeration and diluted at intervals to maintain a titre of approximately 5×10^7 organisms/ml. Samples were removed at intervals for colony counts and for cytological examination. The colony count growth curve thus obtained was identical with that found previously (Maaløe & Lark, 1954). This finding, in addition to the cytological observations to be presented, demonstrated that synchronous nuclear and subsequent synchronous cellular division had indeed been induced.

Samples (4 ml.) were removed for cytological observation and immediately mixed with 1 ml. of a 5 % OsO_4 solution to obtain rapid fixation. After standing for 3 min. at room temperature this solution (now 1 % in OsO_4) was diluted to 40 ml. with broth to avoid overfixation, and centrifuged. Following centrifugation the cells were resuspended in 0·2 ml. broth, and samples of this suspension were immediately spotted on the surface of a dry agar plate. After the samples had dried into the agar surface, the Petri dish was inverted over distilled water and thus kept until impression smears could be made on coverslips. The preparations were hydrolysed for 1 min. in N-HCl at 60° (the previously ascertained optimum time of hydrolysis for nuclear staining of this organism), rinsed in distilled water and stained for 2 hr. at room temperature in 0·25 % (w/v) thionine containing 5 mg./ml. thionyl chloride (DeLamater, 1952). After this the preparations were again rinsed in distilled water, dehydrated in absolute ethanol at −70° for 24 hr., and passed through ethanol (twice) and xylene (three times) at room temperature before being mounted in Canada Balsam.

Control stainings of non-synchronized cultures showed that the liquid-fixation technique, employed to ensure rapid fixation, gave results identical with those obtained by fixation over 1 % osmium tetroxide vapour for 3 min. It should be noted, however, that cells fixed in liquid medium clumped during centrifugation, making it difficult to find fields which were rich in individual cells (cf. Pl. 1, fig. *b*).

Because of the small size of the organisms studied (0·5–0·7 μ. wide, 1–4 μ. long) a Reichert microscope equipped with an 'Anoptral' condenser, a 'Fluorit' objective (×100) and a Plan-ocular (×12) was used. The wide ring-aperture of the condenser (normally used for phase-contrast microscopy with oil-immersion) was adjusted so that light which penetrated the specimen obliquely reached the periphery of the objective leaving the centre region unused. This arrangement, which was suggested by A. Findeis, of the Reichert Company, gives excellent resolution and contrast.

RESULTS

Before and after the shift in temperature used to induce nuclear division, samples were removed from the culture, fixed and stained. In repeated experiments the most striking change noticed when first looking at the various preparations was the large increase in the proportion of quadri-nucleate cells present in the population. This increase began at *c.* 10 min. after the temperature shift, a maximum frequency of quadri-nucleate cells being attained by *c.* 20 min. after the temperature shift. After 20 min. the frequency of quadri-nucleate cells gradually returned to the low value observed before raising the temperature. The return to this state was completed at 50–60 min. after the temperature shift. Pictures of cells fixed before the temperature shift (consisting predominantly of bi-nucleate cells), and of cells from the synchronized population fixed 20 min. after the temperature shift (showing a large proportion of quadri-nucleate cells) are given in Pl. 1, figs. *a* and *b*.

Between the extremes just described more subtle changes in the nuclear bodies were observed. In exponentially growing cultures of *Salmonella typhimurium* at 25° (generation time = 50 min.) or at 37° (generation time = 22 min.) the nuclei appeared predominantly as compact dots, whereas, during the period between the temperature shift and the increase in number of quadri-nucleate cells, a large proportion of the cells showed other nuclear configurations. These configurations could usually be assigned to one of three arbitrary classes designated as wedge, bar and dumbbell structures; the four types of nuclear configuration are illustrated diagrammatically in Fig. 1, and examples of nuclei of the different types are presented on Pl. 2.

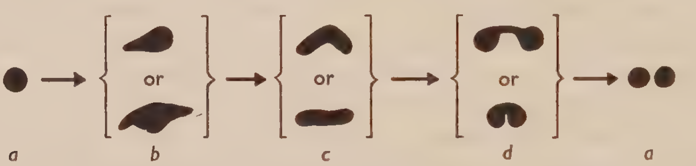

Fig. 1. Schematic representation of the four configurations—dot (*a*), wedge (*b*), bar (*c*) and dumbbell (*d*)—successively assumed by the dividing nucleus of *Salmonella typhimurium*. The two different shapes representing each of the classes *b*, *c* and *d* roughly illustrate the variations observed.

The predominance of dot structures in randomly dividing cultures, as compared to the low frequency found during the period of induced nuclear division, at once suggested that in *Salmonella typhimurium* this configuration represented nuclei not involved in division. Moreover, the preliminary survey of preparations fixed after the temperature shift indicated that the frequencies of bi-nucleate cells containing structures other than dots rose and fell in a systematic way which suggested that nuclear division proceeded from dots via wedge and bar to dumbbell structures. This sequence is illustrated in Fig. 1. With this general impression in mind, an enumeration of cells containing the four different nuclear structures was carried out, using the micrographs obtained with the high-resolution system described above. For practical reasons the cells were classified as follows: (*a*) cells containing 'dots' only; (*b*) cells containing at least one 'wedge' but no 'bar' or 'dumbbells'; (*c*) cells containing at least one bar but no dumbbells; (*d*) cells with one or more dumbbells. The results obtained with this classification are given in Table 1; typical fields showing high frequencies of cells with changed nuclei are presented on Pl. 1, figs. *c* and *d*, which should be compared with Pl. 1, fig. *a*.

For the interpretation of the data in Table 1 it is important to notice that the classification method is biased in favour of cells which contain bars and dumbbells. Thus an increase in the frequency of bar-cells will lower the frequency of wedge-cells for each cell containing a wedge and a bar, while a rise in the frequency of dumbbell cells may depress the frequencies of both wedge- and bar-cells. Conversely, increases in the frequencies of wedge- or bar-cells will be picked up and correctly registered as long as the cells do not at the same time contain bars or dumbbells respectively. These considerations show that the above simple classification may be used to test the hypothesis that during division the nuclear configuration changes from dot via wedge and bar to dumbbell.

Table 1. *Classification of cells according to number of nuclei and to nuclear configuration*

	Bi-nucleate cells				Quadri-nucleate cells				
Time of sampling t	a %	b %	c %	d %	a %	b %	c %	d %	Total of cells scored
−1	74	8	6	3	8	1	0	0	336
1	66	8	7	6	13	0	0	0	246
3	48	20	9	9	12	2	0	0	252
4	41	32	8	8	11	1	0	0	226
5	40	25	16	5	14	0	0	0	183
6	22	33	21	15	6	1	1	1	100
7	15	45	21	9	10	0	0	0	50
8	27	18	17	28	8	1	0	1	272
9	26	16	14	27	16	1	0	0	327
11	30	15	8	35	12	0	0	0	74
13	30	13	12	19	26	0	0	0	146
16	31	7	7	22	27	3	3	0	291
20	23	8	8	22	26	10	2	1	308
25	12	8	5	25	20	22	3	5	190
30	16	9	6	26	25	12	4	2	302
35	27	5	4	33	9	9	5	8	100
60	56	14	8	13	9	0	0	0	209

The cell-classes a, b, c and d are those described on p. 320; viz. cells containing dot, wedge, bar and dumbbell structures respectively. Cells containing two dots and one dumbbell were arbitrarily classified as bi-nucleate class d cells. When comparing the data presented here with previous statements about the high frequencies of quadri-nucleate cells attained around 16–25 min. (Lark & Maaløe, 1954), it should be noted that, previously, bi-nucleate dumbbell cells were scored as quadri-nucleate cells.

Another problem of importance for the interpretation of our data should be mentioned. In order to differentiate between the four types of nuclear configurations it is necessary to work at the limit of optical resolution, which results in considerable differences between the results obtained when different observers try to classify cells according to our scheme. Thus the data presented in Table 1, which were obtained by one of the authors (K.G.L.), differ markedly from similar data obtained by another author (O.M.). However, the two sets of figures differ systematically in such a way that the numbers of wedge, bar and dumbbell structures scored by one observer were consistently higher than those scored by the other. The times when the observed frequencies changed, as well as the magnitudes of these changes, thus seem to be relatively independent of the observer, whereas the absolute values for the frequencies are not.

To bring out clearly the important changes in the frequencies of cells containing dot, wedge, bar and dumbbell structures respectively (the cell-classes *a–d*), data from Table 1 were used to construct Fig. 2 in which the frequencies for bi-nucleate cells for the samples taken until 20 min. after the temperature shift are plotted as functions of time. In addition, the frequencies of quadri-nucleate cells have been plotted. As may be seen the frequency of wedge structures (*b*) began to rise 2 min. after the temperature shift and continued to do so for the next 4 min. Similar increases in bar and dumbbell structures (*c* and *d*) were initiated 2 and 4 min., respectively, after the rise in wedges (*b*). During the increase in dumbbell structures (*d*), the frequencies of wedges (*b*) and bars (*c*) decreased. The steepness of these decreases is undoubtedly due to some extent to the method of classification used; however, the frequency of bi-nucleate cells which contained wedges (*b*) continued to decline after the frequency of dumbbells (*d*) had ceased to increase. This would seem to indicate a real decrease in the frequency of bi-nucleate cells which contain wedge structures.

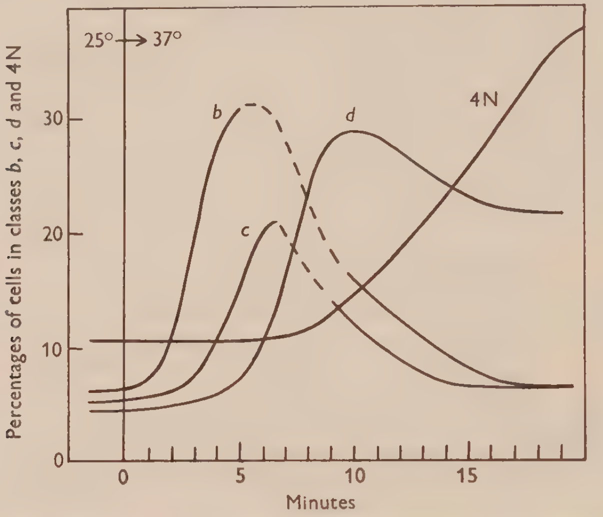

Fig. 2. The changing frequencies of nuclear configurations during the period after the temperature shift. The classes *b*, *c* and *d* are those schematically presented in Fig. 1. Class '4N' is the class of cells with four distinct nuclei. The stippled curve sections indicate that in these regions the shape of curves *b* and *c* are influenced by the simultaneous rise in curves *c* and *d*. (See text on p. 322.)

Finally, at 9–10 min. after the temperature shift, i.e. 2–4 min. following the time at which the frequency of dumbbells (*d*) began to increase, the frequency of cells containing four distinct nuclei began to increase and reached a constant high level within the next 10 min.

From the progression of times at which the frequencies of wedge, bar and dumbbell structures began to increase, and from the time at which the frequency of quadri-nucleate cells began to increase, it may be concluded that the cytological sequence of events during nuclear division in *Salmonella typhi-*

murium is that proposed in Fig. 1. Thus the dot structure, or compact nuclear mass grows and extends thin branches in one or two directions (wedge); the nuclear mass then distributes itself more uniformly (bar), finally coalescing into two terminal globules (dumbbell) which break up to give two compact nuclear masses.

In the period from 16 to 25 min. after the temperature change a slow increase in the frequency of quadri-nucleate cells with wedge structures takes place. An apparent increase in bar and dumbbell structures in quadri-nucleate cells was also noted; a continued high frequency of bi-nucleate dumbbell structures was also seen during this period while, as was mentioned earlier, the frequency of bi-nucleate cells with wedge structures decreased during this period. The only obvious source of bi-nucleate cells containing dumbbells is the quadri-nucleate cells containing wedge structures. This would imply that nuclear division of the quadri-nucleate cells proceeded up to the 'wedge' stage in quadri-nucleate form, at which time the cells divided, and nuclear division was completed in the bi-nucleate stages, thus preserving for some time a high proportion of quadri-nucleate cells. This conclusion is born out by the observation that many quadri-nucleate cells whose nuclei were either in the 'dot' or 'wedge' stage showed transverse dark structures which divided the cell into two bi-nucleate half-cells. These structures, which looked like the septa observed in other cells (e.g. by Murray & Robinow, 1952), were at the limit of the resolving power of the optical system used (see Pl. 1, fig. *d*).

Finally some mention of cell size should be made. In the random cultures at 25 and 37°, the bi-nucleate dot-cells (*a*) were 1–1·5μ. long and 0·5–0·7μ. wide. Following the induction of nuclear division the width of the cells remained fairly constant, but there was steady increase in length of the bi-nucleate cells, the average cell length being 1·2, 1·4, 1·6 and 2·1μ. at 3, 7, 11 and 20 min., respectively. From this it seems that the wedge structure predominantly existed in cells of length 1·4μ. or more, while dumbbells were formed chiefly in cells of length 1·6μ. or more. The length of cells which contained wedge or dumbbell structures, taken from random cultures, agreed with this idea.

DISCUSSION

The foregoing studies were undertaken in an effort to elucidate the sequence of visible changes during nuclear division. It was felt that by studying a bacterial population in which synchronous nuclear division had been induced, it would be possible to recognize definite cytological stages in the division process. This attempt has met with only a limited success. A crude series of successive cytological stages has been ascertained for nuclear division in *Salmonella typhimurium*. These stages are represented in Fig. 1 as 'dot', 'wedge', 'bar' and 'dumbbell' structures, and this classification could not be refined because of the limited resolution of the optical system used. Indeed, as has been pointed out, many borderline cases have been observed, making unequivocal classification of the nuclear configurations impossible. As may be seen from Pl. 2, the 'bar' class contains transition stages between 'wedge' and 'dumbbell' configurations.

From the data to hand, it seems that the most reasonable description of the sequence in nuclear division in *Salmonella typhimurium* is as follows: nuclear division can be pictured as the gradual transition from one compact body via the more irregular structures described as 'wedge', 'bar' and 'dumbbell' into two distinct round bodies; this picture would tend to agree with that advanced by Robinow (1946). Nuclear configurations of the types claimed by DeLamater (1951) to represent mitotic patterns were observed occasionally. However, no accumulation of such configurations was found during the transition from the bi- to the quadri-nucleate state.

The elucidation of the detailed mechanism of the transition from the two- to the four-dot stage must await studies by means of electron microscopy. This end would seem to be much advanced by the present studies. As has been shown (Birch-Andersen, 1955) serial sections of *Salmonella typhimurium* may be superimposed on one another to permit a high resolution composite view of the cell. From the overall appearance of such a composite picture, it should be possible to establish whether the nucleus in question is of a dot, wedge, bar or dumbbell configuration and thus roughly establish its position in the sequence of nuclear division. Based on this procedure for identification of the nuclear state an analysis of the individual sections may permit a detailed mapping of the nuclear structures.

Because of the lack of precision in differentiating between the dot, wedge, bar and dumbbell configurations it is difficult to correlate the cytological changes observed after the induction of nuclear division with physiological changes, such as the ability to become lysogenized upon infection with a temperate phage (Maaløe & Lark, 1954). Nevertheless, certain qualitative comparisons may be made. In Fig. 3 the characteristic change in the lysogenization frequency is illustrated by the fully drawn curve (1) which is to be compared with the dotted curves (2 and 3) which show the changes in the cumulative frequencies of bi-nucleate cells containing nuclei representing early and late stages of division respectively; viz. curve 2 which shows the cumulative frequencies of cells containing at least one nucleus which is not of the dot type, and curve 3 which shows the frequencies of cells containing at least one dumbbell. As Fig. 3 shows, curve 2 rises first and this rise is soon followed by the increase in lysogenization frequency, whereas the rise in curve 3 sets in 2–3 min. later. We previously advanced the hypothesis that the doubling of the number of nuclei/cell is reflected, physiologically, in a doubling of the lysogenization frequency (Maaløe & Lark, 1954). In the light of the cytological evidence now at hand we may conclude that soon after a nucleus has assumed the wedge configuration it reaches a state in which neither the process of lysogenization nor lowering of the temperature to 25° (at which temperature phage infection takes place) interfere with nuclear division.

Finally we would like to discuss the septate appearance of quadri-nucleate cells during the period in which the colony count rises abruptly. As has been mentioned, such 'septa' appear on the limit of resolution of the optical system and the difficulties encountered in seeing them might be due to a failure to employ cell-wall stains. However, it is of interest to note that no structure of a septum-like nature has been observed in electron micrographs of sectioned cells taken from this period of a synchronized typhimurium system, although many cells have been observed in various stages of cellular division. (Ranging from a slight pinching to an almost complete severance of the cell, by constriction, into two cells; Birch-Andersen, Lark, Maaløe, Sjöstrand, 1954.)

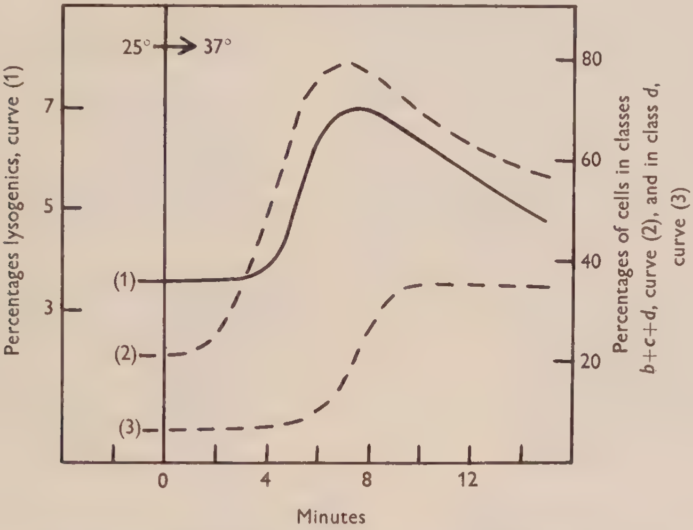

Fig. 3. The frequency of lysogenization compared with the frequencies of cells with actively dividing nuclei. Curve 1, illustrating the abrupt rise in the frequency of lysogenization, is transcribed from a previous paper (Maaløe & Lark, 1954). Curve 2 illustrates the rise in the cumulated frequency of bi-nucleate cells containing wedge, bar or dumbbell structures, i.e. cells with actively dividing nuclei. Curve 3 represents the rise in the frequency of bi-nucleate cells with one or two dumbbells.

From this, it seems that the septate appearance of the cell is not due to a septum being formed across the cell before division but is due to some optical artefact, which arises because of the constricted character of the cell. True septa have been demonstrated conclusively by means of electron microscopy of thin sections (Chapman & Hillier, 1953) in *Bacillus cereus* which, however, possesses a thick and clearly differentiated cell wall which is altogether lacking in *Salmonella typhimurium.* In view of this, it may be wise to re-examine by means of thin section electron microscopy many of the septum-type structures reported in the literature.

We are grateful to Dr N. O. Christensen for the loan of his microscopical equipment.

Our best thanks are due to Doctors M. H. Adams, W. L. Barksdale and B. A. D. Stocker, who read the manuscript and suggested important improvements. One of us (K. G. L) is a Fellow of the American Cancer Society, upon recommendation of the Committee on Growth, National Research Council.

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EXPLANATION OF PLATES

Plate 1: *Salmonella typhimurium*.

Cells fixed and stained selectively for DNA at different times relative to the temperature shift. Magnification, ×3400.

Fig. *a*. Predominantly bi-nucleate cells, as seen in a culture growing exponentially at 25°. Cells with 'dots' only, class *a*.

Fig. *b*. Predominantly quadri-nucleate cells, as seen 20 min. after raising the temperature to 37°. Note the unfortunate tendency to clumping after centrifugation of the fixed cells. Cells with one or more 'wedges', class *b*.

Figs. *c*, *d*. Cells fixed 11 and 30 min. after the temperature shift, respectively. Fields showing many cells with actively dividing nuclei, i.e. nuclei of the classes *b*, *c* or *d*, illustrated on Pl. 2. Cells marked with ↗ contain 'bars', class *c*. Cells with one or more 'dumbbells', class *d*.

Plate 2

Examples of cells of classes *a*, *b*, *c* and *d*; see p. 320–321. Magnification, ×5100.

(*Received* 22 *March* 1955)

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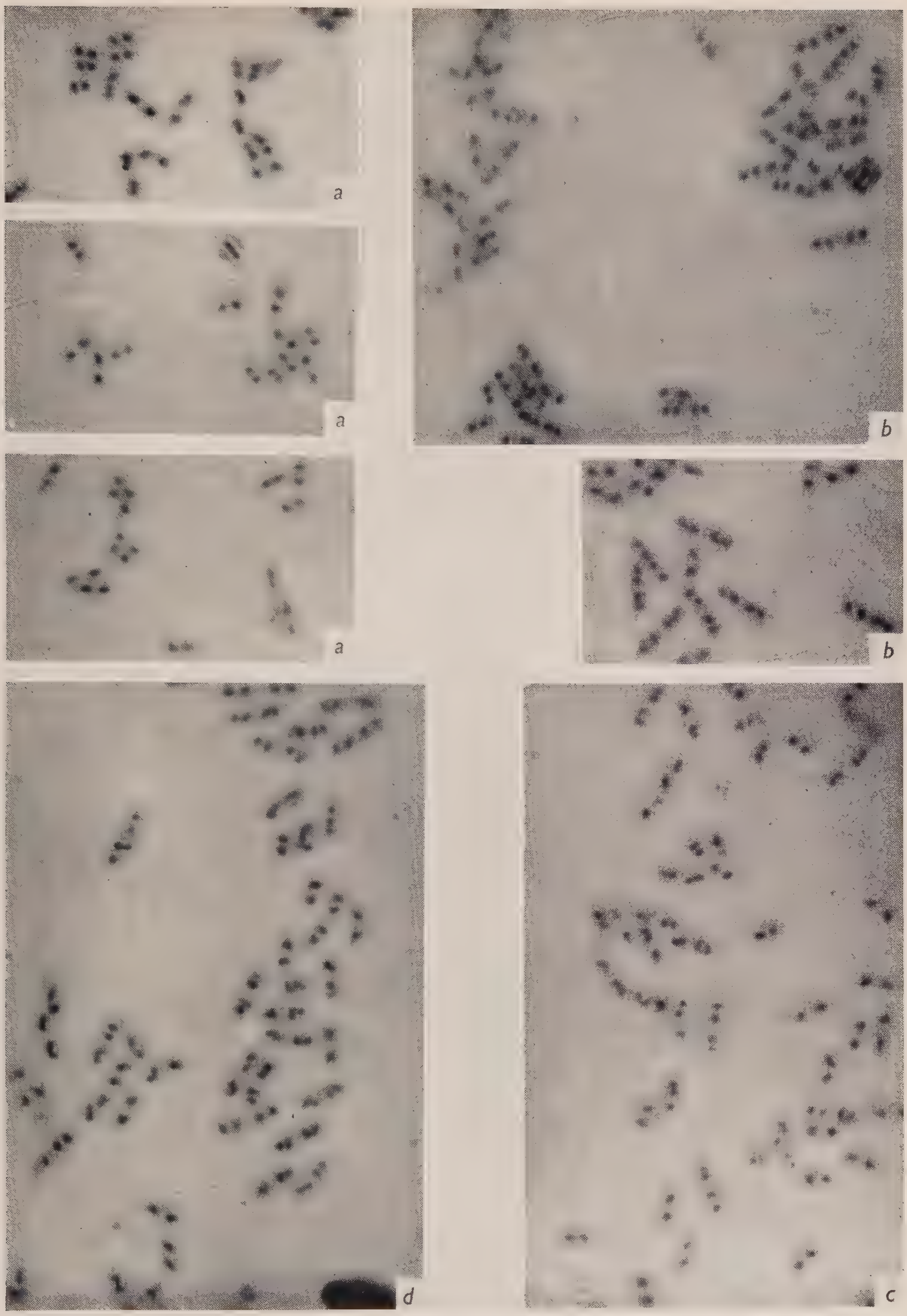
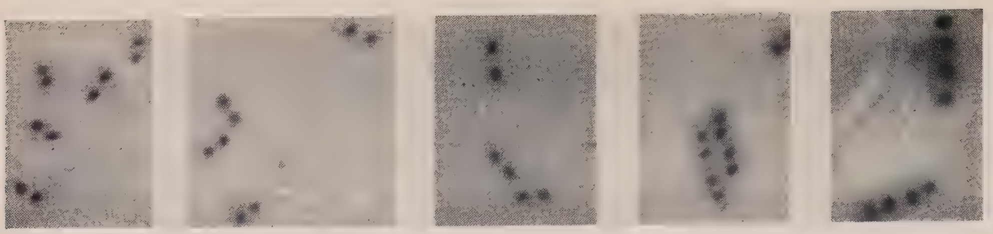
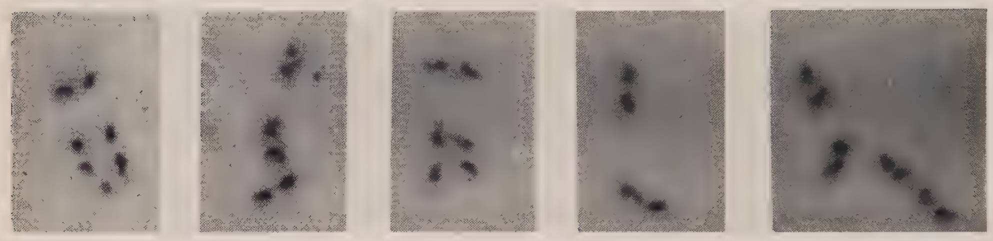


K. G. Lark, O. Maaløe & O. Rostock—Nuclear division in *S. typhimurium*. Plate 1

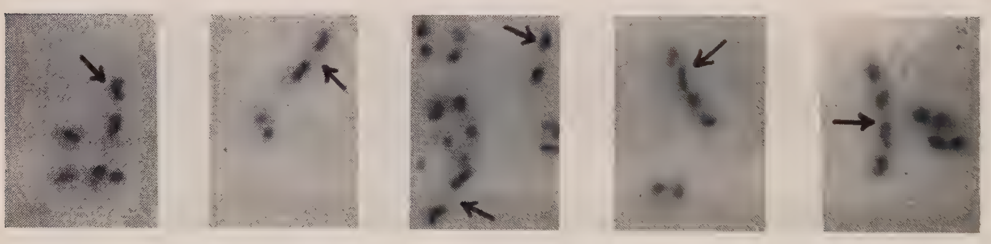
(*Facing p.* 326)



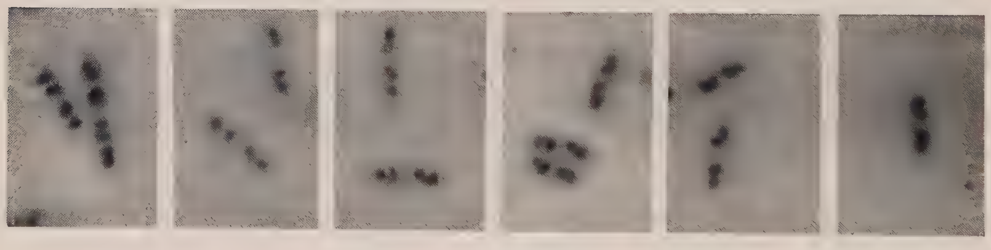
(*a*) Cells with dots only, class a.



(*b*) Cells with one or more wedges, class b.



(*c*) Cells marked with ↗ contain bars, class c.



(*d*) Cells with one or more dumbbells, class d.

K. G. LARK, O. MAALØE & O. ROSTOCK—NUCLEAR DIVISION IN *S. TYPHIMURIUM*. PLATE 2

Birch-Andersen, A. (1955). *J. gen. Microbiol.* **13**, 327–329

Reconstruction of the Nuclear Sites of *Salmonella typhimurium* from Electron Micrographs of Serial Sections

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SUMMARY: A method for obtaining a composite picture of the organisms from electron micrographs of serial sections is described. Evidence is presented that the same general structures are revealed, as far as the nuclear sites are concerned, by selective staining for deoxyribonucleic acid and by electron microscopy of sections.

Studies of electron micrographs of sections of cells fixed at various times during synchronous division do not permit the identification of characteristic configurations which suggest successive stages of nuclear division (Birch-Andersen, Lark, Maaløe & Sjöstrand, 1954). The probable reason for this is that the integrated picture of the cell is lost when individual thin sections are studied. In principle, this difficulty has been overcome by a method now developed for obtaining serial sections from which a composite picture of the cell can be constructed. However, the labour involved in this process makes the study and classification of large numbers of cells impracticable, and we have therefore investigated whether the nuclear sites, as they appear in composite pictures, may be identified as belonging to one of the configurations (dot, wedge, bar or dumbbell) described in the preceding paper (Lark, Maaløe & Rostock, 1955). Preliminary studies now to be described indicate that this is possible, and that the state of a cell with respect to nuclear division may be judged from the shape of the nuclear vacuoles. If this be substantiated, a study of the structural details of the dividing nucleus by means of electron microscopy of thin sections may be undertaken.

METHODS

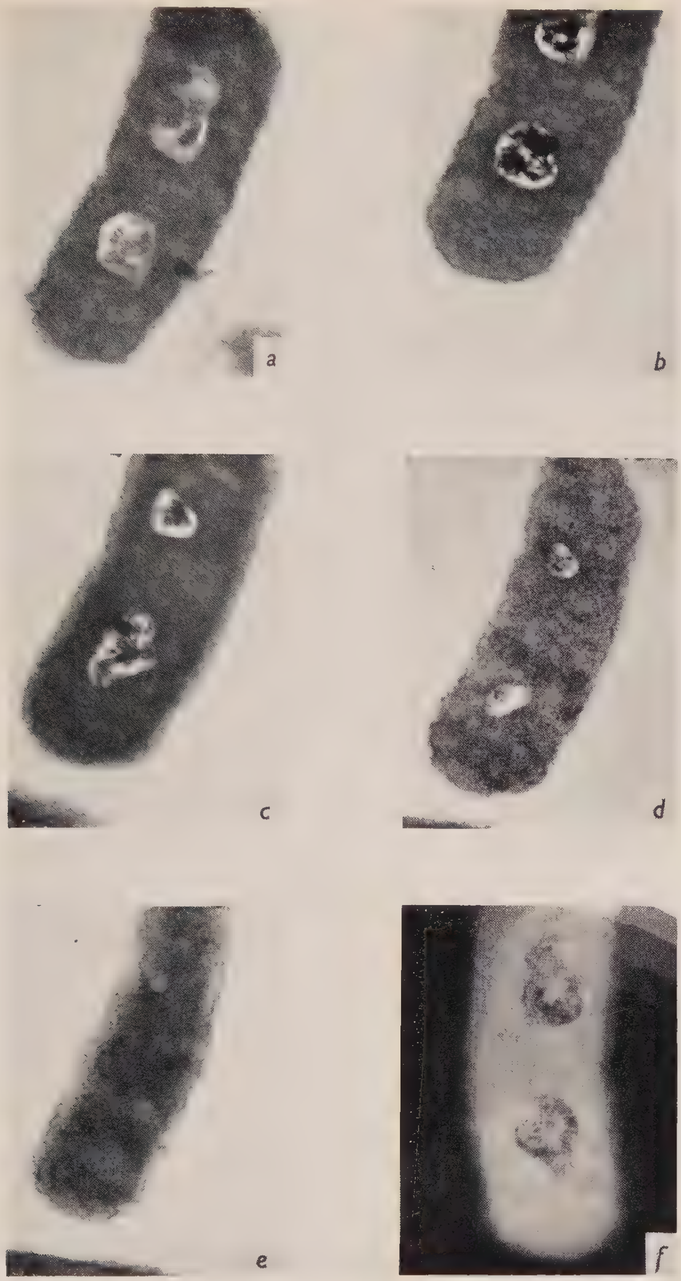
Fixation, dehydration, embedding and sectioning were performed as described by Birch-Andersen, Maaløe & Sjöstrand (1953); the procedure for obtaining serial sections of bacteria by means of the Sjöstrand ultramicrotome will be described in a later publication. Electron micrographs were taken with the Philips E.M. 100 electron microscope equipped with the new 25 A. objective lens. The standard objective aperture, as delivered by the factory, was used. Exposures were made at 60 kV., with a primary magnification of about ×4300 on 35 mm. film (Kodak Fine Grain Positive Film), using a low beam intensity and without removing the embedding material.

For the present investigation some thirty organisms were selected, from each of which a complete series of longitudinal sections had been obtained. Composite pictures of each of these cells were made as follows. A series of positive contact prints was made on a 35 mm. film, and the exposure time needed for producing five times enlarged negative prints on paper determined. Usually, the first as well as the last section in a series show no traces of nuclear sites in the bacterial cytoplasm; such sections were omitted in the rest of the procedure. This consisted of printing the remaining sections, one on top of the other, on to the same piece of photographic paper. The exposure time for each section was made equal to the predetermined time divided by the number of sections. So far no attempt has been made to estimate the thickness of the individual sections and to adjust exposure times accordingly. Between each exposure the outline of the cell was traced to permit the outline of the next section to be fitted to that of its predecessor. By this procedure we end with composite pictures of the kind shown on Pls. 1 & 2, which also show the series of individual sections, printed in positive, from which the reconstructions were made.

RESULTS

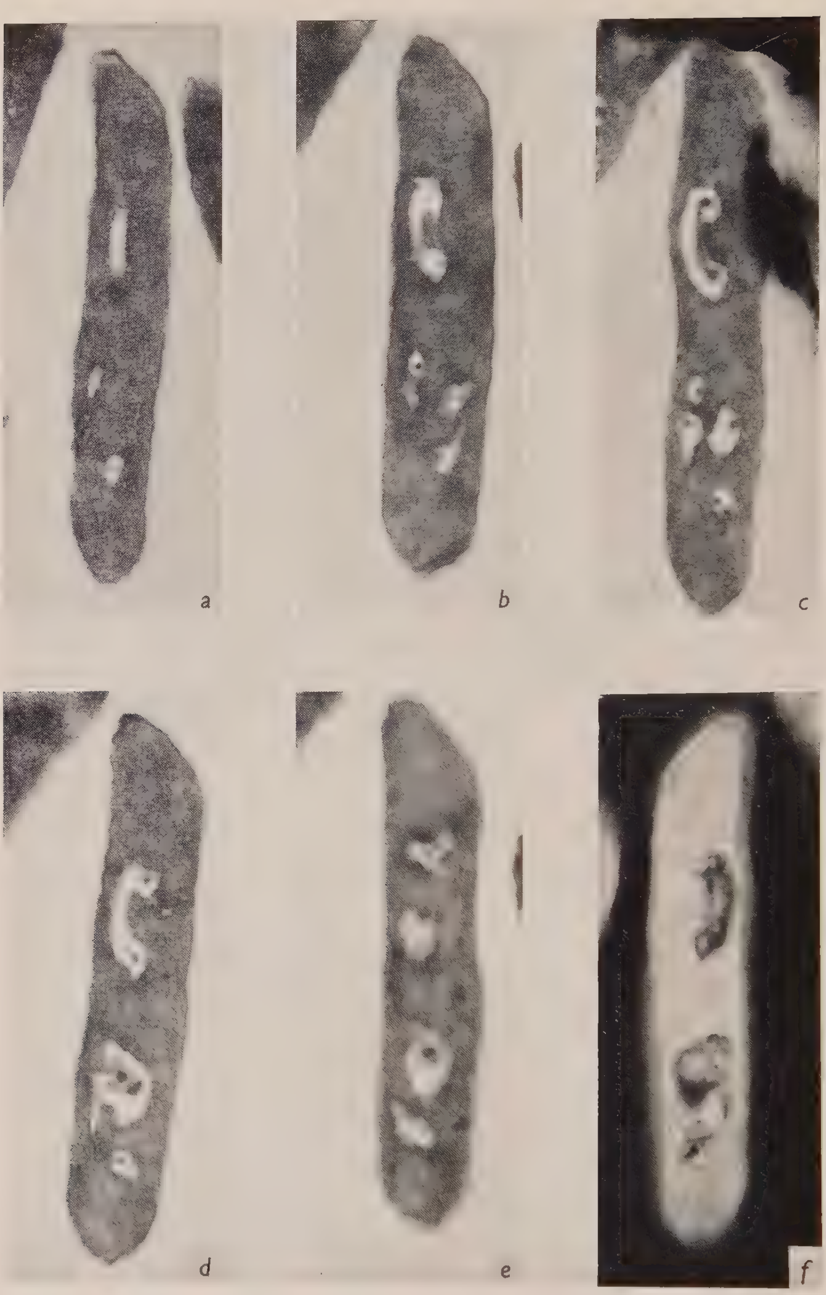
In the composite picture, printed in negative, the nuclear sites appear as black 'vacuoles' whose contents, as well as the bacterial cytoplasm, are white. A striking feature of the composite pictures is that, despite the irregularity in shape and distribution of the light areas in some of the individual sections, the reconstructions show that these areas combine to form definite nuclear regions (2, 3 or 4/cell) which seem not to communicate. The reconstructions presented here show nuclear regions which resemble the configurations designated by Lark *et al.* (1955) 'dot', 'wedge' and 'dumbbell' (Pl. 1, fig. *f* 'dot'; Pl. 2, fig. *f* 'wedge' and 'dumbbell'). The relatively high resolution in the composite pictures accounts for the fact that the outlines of the nuclear sites appear more irregular in the electron micrographs than in the photographs of stained cells. By means of the composite picture technique it has been possible to identify the types of nuclear sites shown in Pl. 1 and 2, and the bar type, described by Lark *et al.* (1955), has also been seen in some cases. It should be emphasized that about 30% of the nuclear sites so far examined by this technique could not be classified as belonging to one of the four configurations mentioned; however, these uncertain cases did not include configurations which could not be considered as intermediate types. As might be expected, in the composite pictures the most uncertain distinction is that between the dot and the wedge configurations.

The observations that the 'holes' or 'vacuoles' seen in individual sections combine to form closed regions and that the outlines of these regions resemble the nuclei as they appear in preparations stained selectively for deoxyribonucleic acid (DNA) strongly suggest that the two methods reveal the same general structures. It is not clear, however, whether the stained areas in Pls. 1 and 2 of the paper by Lark *et al.* (1955) correspond to the entire nuclear region as seen in the composite pictures of this paper or to the dense material inside these regions. It cannot be excluded, at the present time, that the fixation and dehydration processes which precede embedding, cause the DNA



A. Birch-Andersen—Reconstructions from electron micrographs. Plate 1

(*Facing p.* 328)



A. Birch-Andersen—Reconstructions from electron micrographs. Plate 2 contained in the nuclear regions to shrink, or that acid hydrolysis and staining cause swelling of the DNA to make it fill the nuclear vacuoles more or less completely. For future studies of the structural details of the dividing nucleus it is, of course, essential to establish whether such artefacts are introduced during the preparation of the specimens. Control experiments comprising different fixation conditions and combination of staining and sectioning procedures, now being carried out in this laboratory, may settle these questions.

Special thanks are due to Mrs H. Ravn for valuable assistance in working out technical details of the procedure for collecting serial sections, and to Miss A.-G. Overgaard for carrying out the difficult photographic work involved in the making of the composite pictures.

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EXPLANATION OF PLATES

Plate 1

Electron micrographs of individual sections of a *Salmonella typhimurium* cell (figs. *a–e*), and a composite picture (fig. *f*) produced as described on p. 328. Note that fig. *f* is a negative and, therefore, a mirror image of the individual sections. Section *e* was not used in making the composite picture, since no 'nuclear vacuoles' have been sectioned in this case. Magnification, ×21,500.

Plate 2

Individual sections and composite picture, as on Pl. 1. Here all sections were used to make the picture of fig. *f*. Magnification, ×21,500.

(*Received* 22 *March* 1955)

Archetti, I., Donald, H. B., Isaacs, A. and Valentine, R. C. (1955). *J. gen. Microbiol.* 13, 330–337

The Effects of Metaperiodate and Heated Influenza Virus on Influenza Virus Growth

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and R. C. VALENTINE

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SUMMARY: Appropriate quantities of sodium metaperiodate injected into hen eggs inhibited the development of full infectivity by some strains of influenza virus, i.e, 'incomplete' virus was produced; with other strains of virus the same quantities of metaperiodate inhibited haemagglutinin production. Metaperiodate in a dose which of itself did not inhibit haemagglutinin production by two strains of influenza virus enhanced the inhibitory action of heated influenza virus on haemagglutinin production by these strains. Both sodium metaperiodate and heated influenza virus inhibited the production of influenza virus filaments more than that of spheres. It is suggested that both metaperiodate and heated influenza virus act on a common substrate which is required for the development of both viral agglutinin and full virus infectivity.

The growth of influenza virus in the chick embryo can be inhibited in two distinct ways; by partially or by wholly inactivated influenza virus. Firstly, virus wholly inactivated by heat at 56° or by ultraviolet irradiation can cause inhibition of haemagglutinin production by influenza virus (Henle & Henle, 1944); in this situation there is an inhibition in the yield of virus particles, but any particles produced are normally infective. Secondly, serial passages of influenza virus at low dilution (von Magnus, 1946), or the use of virus partially inactivated at 37° (Horsfall, 1954) cause the production of virus with a low infectivity relative to the haemagglutinin titre ('incomplete' virus); in this case there is usually no diminution in the number of virus particles produced, but each particle has a very low probability of initiating infection. These two reactions are usually considered to be unrelated; they may be thought of as inhibitory actions on different stages of virus growth. von Magnus (1954) summarized the evidence for the hypothesis that infectivity is acquired late in virus development, and that 'incomplete' virus is immature in the sense that it is released from the cells without the virus having acquired the property of infectivity.

Recently, Fazekas de St Groth & Graham (1954*a*) showed that in eggs treated with sodium metaperiodate and then inoculated with influenza virus, the virus produced had a low infectivity relative to the haemagglutinin titre, and thus corresponded to the incomplete virus of von Magnus (1946). These workers also showed that different strains of influenza virus varied in the ease with which they could be induced to form incomplete virus by serial passages at low dilution; the virus strains could be arranged in order of ease, and this order seemed to correspond roughly to a gradient of the affinity of the viruses for chorio-allantoic membrane receptor substance (Fazekas de St Groth &

Graham, 1954*b*). This was of great interest to us since previous results had suggested a similar correspondence between the affinity of viruses for chorio-allantoic membrane receptor substance and the ease with which interference by heated influenza virus could be induced (Isaacs & Edney, 1951). These findings suggested the possibility that the two inhibitory phenomena might be related. We have investigated the effects of sodium metaperiodate on different strains of influenza virus and our results, reported in this paper, show that an amount of sodium metaperiodate which inhibits the development of infectivity in one strain may inhibit agglutinin production in another. This supports the idea that the two inhibitory phenomena may be related. It will also be shown that there are some similarities in the action of sodium metaperiodate and heated influenza virus when tested as inhibitory reagents for different influenza virus strains.

MATERIALS AND METHODS

Virus strains. Influenza viruses PR8, MEL and A/Persian Gulf/2/52 were kept in capillary tubes at −70°. A/Persian Gulf/2/52 was used as 3rd or 4th egg passage material. Batches of virus were prepared by inoculating 10-day eggs by the allantoic route with 10^3 to 10^4 50% egg-infective doses (EID50) of seed virus and harvesting after incubation for 42 hr. at 35°.

Agglutination titrations. Agglutination titrations were carried out by a pattern test in plastic plates and the results expressed as the reciprocal of the initial dilution of virus at the partial agglutination end point. One agglutinating dose is defined as the least amount of virus required to agglutinate 0·25 ml. of a 1% suspension of washed chick red cells (about $10^{7\cdot1}$ to $10^{7\cdot2}$ cells).

Infectivity titrations. Serial 3·16-fold ($\sqrt{10}$) dilutions of virus were prepared in broth-saline (50% Hedley Wright broth in normal saline) and 0·05 ml. volumes inoculated into the allantoic sac of 10-day-old chick embryos, which were harvested after 72 hr. incubation at 35° and tested for agglutinins. The end-points were calculated by the method of Reed & Muench (1938) and the results expressed as EID50/ml.

Experiments with metaperiodate. Eggs were treated with metaperiodate as described by Fazekas de St Groth & Graham (1954*a*). Chick embryos (10-day) were inoculated in the allantoic sac with 1 ml. of 0·01 M-sodium metaperiodate. One hour later the excess metaperiodate was neutralized by an intra-allantoic inoculation of 0·25 ml. 0·1 M-glycerol, and these eggs and control eggs were inoculated 2 hr. afterwards with the virus under test. The eggs were harvested after incubation for a further 42 hr. at 35° C.

Interference experiments. Chick embryos (10-day) were inoculated in the allantoic cavity with inactivated influenza virus, as allantoic fluid heated at 56° for 1 hr. 24 hr. later these and control eggs were inoculated with the virus under test and the eggs were harvested after a further 42 hr. incubation at 35°.

Virus counts on red cells. The numbers of virus filaments and spheres found in samples of allantoic fluid harvested from the treated eggs were counted on electron micrographs after adsorption on red cell membranes (Dawson & Elford, 1949). The concentration of red cell membranes used for a particular absorption was dependent on the haemagglutinin titre of the virus examined. The allantoic fluid was spun lightly to remove debris, mixed with the laked red cell suspension and kept for 1 hr. at 5°. The cells were then fixed with 0·1 % osmium tetroxide for 30 min., spun down and washed with distilled water at 5°, mounted on electron microscope grids and shadowed with gold manganin. Electron micrographs were taken of twelve or more red cells from each experiment at a magnification of about 5000 times and the numbers of filaments and spheres were counted and totalled for all the cells. The percentage of filaments in the sample was thus found. Assuming a random distribution of the two forms of virus, the standard error in this percentage will be that of a binomial distribution and is given by $\sqrt{(SF/N)}$, where N is the total number of virus particles, and S and F the percentages of spheres and filaments.

RESULTS

Effect of sodium metaperiodate on the ratio of infectivity to agglutinin in three strains of influenza virus

Fazekas de St Groth & Graham (1954*a*) found that, in eggs treated with sodium metaperiodate and subsequently inoculated with high dilutions of the PR8 and Lee strains of influenza virus, the virus produced had a significantly lower ratio of infectivity to agglutinin titre than when grown in normal untreated eggs. Our results in experiments of this type have been very variable with different strains of virus. Table 1 shows the ratios of infectivity to agglutinin titres of individual eggs inoculated with the MEL, PR8 and A/Persian Gulf/2/52 viruses after pre-treatment with sodium metaperiodate.

Table 1. *Effect of sodium metaperiodate treatment of eggs on the ratio (log) of infectivity to haemagglutinin titres in three strains of influenza virus*

Strain	Inhibitory agent	Individual ID50/HA ratios (log)	Mean ID50/HA ratio
MEL	Metaperiodate	5·5, 5·2, 5·0, 6·3, 6·0, 5·9	5·65
	Nil—control	5·7, 5·9, 6·1, 6·2, 6·3, 5·7, 5·8	5·96
PR 8	Metaperiodate	5·1, 4·9, 4·8, 4·9, 5·6, 4·9	5·03
	Nil—control	5·6, 4·7, 5·0, 5·2, 4·9, 4·6, 5·2	5·03
A/Persian Gulf/2/52	Metaperiodate	5·6, 5·4, 5·4	5·47
	Nil—control	4·9, 5·8, 5·6, 5·4, 5·3	5·4

Table 1 shows that metaperiodate treatment of the eggs has not induced any striking reduction in the ratio of infectivity to agglutinin titres of these viruses. The differences from the results of Fazekas de St Groth & Graham (1954*a*) should be viewed in the light of two facts.

(*a*) The normal ratio of infectivity to agglutinin titre is about $10^{6\cdot 0}$ when calculated as described in Methods. Our PR8 and A/Persian Gulf/2/52 strains therefore show, in untreated eggs, ratios which are significantly lower than those of normal strains. Possibly, under those conditions, metaperiodate treatment of eggs does not reduce the ratio further.

(*b*) With all three strains, but particularly with A/Persian Gulf/2/52 virus, metaperiodate treatment has caused a significant drop in haemagglutinin production in some eggs. This finding was not observed in the studies of Fazekas de St Groth & Graham. In this connexion, the results with MEL virus are interesting. In most experiments with this strain, metaperiodate treatment did not reduce the yield of virus haemagglutinin, and the first three results with this strain, shown in Table 1, indicated a slight drop in the ratio of infectivity to agglutinin titres on treatment with metaperiodate. In a later experiment, there was a significant drop in the yield of haemagglutinin with MEL virus, but the virus produced had a normal ratio of infectivity to haemagglutinin (see the last three results with MEL virus in Table 1). Our results suggest that metaperiodate treatment of eggs causes either a drop in the ratio of infectivity to agglutinin titres, or a reduced yield of haemagglutinin but not both effects at once.

Effect of metaperiodate on the yield of virus haemagglutinin

At different times metaperiodate has caused a significant drop in the yield of haemagglutinin with all three strains of influenza virus which we have investigated, but the effect was most regular with A/Persian Gulf/2/52 virus. The results of a characteristic experiment are shown in Table 2.

Table 2. *Effect of sodium metaperiodate treatment of eggs on the production of haemagglutinin by A/Persian Gulf/2/52 virus*

Inhibitory agent	Haemagglutinin titres of individual eggs
Metaperiodate	240, 5, 5, 30, 60, <1, 5, 100, <1, 120, 100, 15, <1, 30, 40, 80
Nil—control	240, 320, 60, 240, 320, 320

Table 2 shows haemagglutinin titres of individual periodate-treated eggs inoculated with A/Persian Gulf/2/52 virus and compared with untreated control eggs. The periodate-treated eggs show big variations in titre, from complete suppression of haemagglutinin production to no apparent effect when compared with the controls.

It was found that the metaperiodate action could be reversed by increasing the dose of virus; when undiluted virus (about 10^7 EID50) was used there was no depression of haemagglutinin production. The reason for this finding is not at present clear.

Combined action of metaperiodate and heated influenza virus on the yield of virus haemagglutinin

In most of our experiments with PR8 and MEL viruses, metaperiodate did not reduce the yield of virus haemagglutinin. However, when metaperiodate treatment, which of itself did not affect haemagglutinin yield, was combined with inoculation of heated influenza virus, the inhibitory effect of the latter was greatly increased. In order to show this, it was necessary to give the metaperiodate after the heated influenza virus; given before, this effect was absent. The result of an experiment which shows the combined action is given in Table 3.

Table 3. *Effect of the combined action of sodium metaperiodate and heated Lee virus on haemagglutinin production by PR8 virus*

Inhibitory agent	Haemagglutinin titres of individual eggs
2560 A.D. of heated Lee	1280, 80, 640, <1, <1
Metaperiodate	1600, 2560, 640, 2560, 2560, 2560
2560 A.D. of heated Lee and metaperiodate	8, <1, <1, <1, <1, <1
Nil—control	2560, 480, 2560, 1280, 2560, 1280

The metaperiodate, which of itself did not inhibit haemagglutinin production by PR8 virus in this experiment, enhanced the inhibitory action of heated Lee virus. This type of result has been obtained in many experiments with PR8 and MEL used as challenge viruses and heated PR8, MEL or Lee used as interfering viruses. We have not carried out experiments of this kind with A/Persian Gulf/2/52 virus, since this strain is much more sensitive than PR8 or MEL, both to the action of metaperiodate, and to interference by heated influenza virus.

Effect of heated influenza virus and metaperiodate on production of influenza virus filaments

The bulk of the evidence available strongly supports the view that heated influenza virus inhibits virus growth at an intracellular site, i.e. the effect is on the virus multiplication within the cell (e.g. Henle, 1953). The fact that metaperiodate may inhibit the development of viral haemagglutinin or infectivity in different strains raised the possibility that this agent might also be blocking different stages of virus production within the cell. One method of gaining further information on this point was suggested by the known properties of influenza virus filaments. It has been shown that in the A/Persian Gulf/2/52 virus, filaments contain a higher proportion of haemagglutinin to infectivity than spheres, i.e. each filament has about the same probability of initiating infection as a sphere, but has a much greater efficiency in agglutinating red cells (Donald & Isaacs, 1954). Any agent which inhibited the production of viral haemagglutinin might therefore be expected to depress filament production to a greater extent than production of spheres.

Preliminary experiments to test this point showed that when eggs were inoculated with A/Persian Gulf/2/52 virus after a preceding inoculation of heated influenza virus, virus filaments appeared to be scanty on electron or dark-ground microscopy. In order to obtain a quantitative estimate of the extent to which filament production was inhibited by heated influenza virus or metaperiodate relative to sphere production, the numbers of filaments and spheres adsorbed on suitable red cells were counted. For differentiating, a rod which was more than twice as long as it was broad was counted as a filament, and filament length was not taken further into account. The results of some counts are shown in Table 4.

Table 4. *Effect of sodium metaperiodate and heated influenza virus on the percentage of filaments produced by A/Persian Gulf/2/52 virus*

Exp. no.	Inhibitory agent	Haemagglutinin titre of fluid examined	No. of particles counted	Percentage of filaments
1	160 A.D. of heated MEL	120	600	4 ± 1
	Nil—control	640	600	50 ± 2
2	640 A.D. of heated PR 8	60	500	8 ± 1
	Nil—control	480	500	43 ± 2
3	Metaperiodate	15	263	11 ± 2
	Nil—control	640	551	36 ± 2
4	Metaperiodate	30	655	7 ± 1
	Nil—control	320	566	22 ± 2

It is clear that there has been a pronounced drop in the proportion of filaments to spheres as a result of pre-treating eggs with either sodium metaperiodate or heated influenza virus, and the differences from the controls are highly significant ($p < 0{\cdot}001$) in each experiment. It seemed unlikely that this finding was due to the low agglutinin titres *per se*, since filaments are more efficient at agglutinating red cells than spheres and simple dilution should, if anything, increase the proportion of filaments. An experiment carried out to test this point showed no significant change in the proportion of filaments on diluting a preparation of virus ten times before adsorbing on to red cell ghosts.

The results of electron microscopy are illustrated in Pl. 1, figs. 1–4. There are roughly the same numbers of virus particles on each red cell, since the numbers of red cells used for the absorption were based on the agglutinin titre of each preparation. However, Pl. 1, fig. 1, shows that interference by heated influenza virus results in a decreased proportion of virus filaments to spheres compared with fig. 2 illustrating the corresponding control preparation; fig. 3 shows the effect of metaperiodate treatment, and again there is a decreased proportion of virus filaments to spheres compared with the corresponding control preparation in fig. 4.

DISCUSSION

These results show some interesting similarities in the inhibitory actions of heated influenza virus and sodium metaperiodate on the growth of influenza virus in the allantoic cavity of the chick embryo.

(*a*) A single dose of sodium metaperiodate may inhibit the development of full virus infectivity (i.e. cause the production of incomplete virus) in some strains, but inhibit haemagglutinin production in other strains. Similarly, influenza virus heated for 1 hr. at 56° may inhibit the development of full virus infectivity without affecting haemagglutinin production (Horsfall, 1954); with larger doses of heated influenza virus it is more usual to find an inhibition of haemagglutinin production. In a number of investigations with these two inhibitory reagents, it has been found that there is either a decreased number of virus particles produced (inhibition of agglutinin production) but each particle produced is normally infective; or there is a normal number of agglutinating particles produced but with reduced infectivity (i.e. incomplete virus).

(*b*) Sodium metaperiodate which of itself does not inhibit haemagglutinin production, enhances the inhibitory action of heated influenza virus.

(*c*) Both sodium metaperiodate and heated influenza virus inhibit the production of virus filaments more than that of spheres.

(*d*) A single dose of sodium metaperiodate inhibits the development of virus infectivity of different strains to a varying extent, corresponding with the affinity of different virus strains for chorio-allantoic membrane receptor substance. There appears to be a similar relationship for the inhibitory action of heated influenza virus on haemagglutinin production by different virus strains.

One theory which might cover these different observations is that there is a factor which is required for both the development of full virus infectivity and for the development of viral haemagglutinin, and that sodium metaperiodate and heated influenza virus owe their inhibitory activity to their ability to compete for this factor. Presumably, different strains vary in their requirements for this factor, and in any strain more factor is required for the development of full virus infectivity, since the development of infectivity is inhibited more readily than that of haemagglutinin. Such a factor (or factors) is unknown but one possibility which suggests itself is chorio-allantoic membrane receptor substance. This is known to be present in the chorio-allantoic membrane, but diminishes greatly during the first 24 hr. of influenzal infection (Edney & Isaacs, 1950; Liu & Henle, 1951). Subsequent increases and decreases in the content of membrane inhibitory titre coincide with decreases and increases respectively, in the rate of production of influenza virus from the membrane (Schlesinger, 1953). Heated influenza virus combines readily with chorio-allantoic membrane inhibitor and metaperiodate inactivates it. There is also evidence that this substance is required for the growth of influenza virus in the chick chorion (Depoux & Isaacs, 1954). It is interesting that Ackermann & Massaab (1954) have suggested that the action of influenza virus enzyme on this substrate may be necessary to complete the final stages of the release of fully infective virus from susceptible cells. It is suggested, therefore, that metaperiodate and heated influenza virus compete with the enzyme of active influenza virus for chorio-allantoic membrane inhibitor; and that this is the basis for their inhibitory effect on the development of haemagglutinin or of full virus infectivity by different strains of influenza virus.

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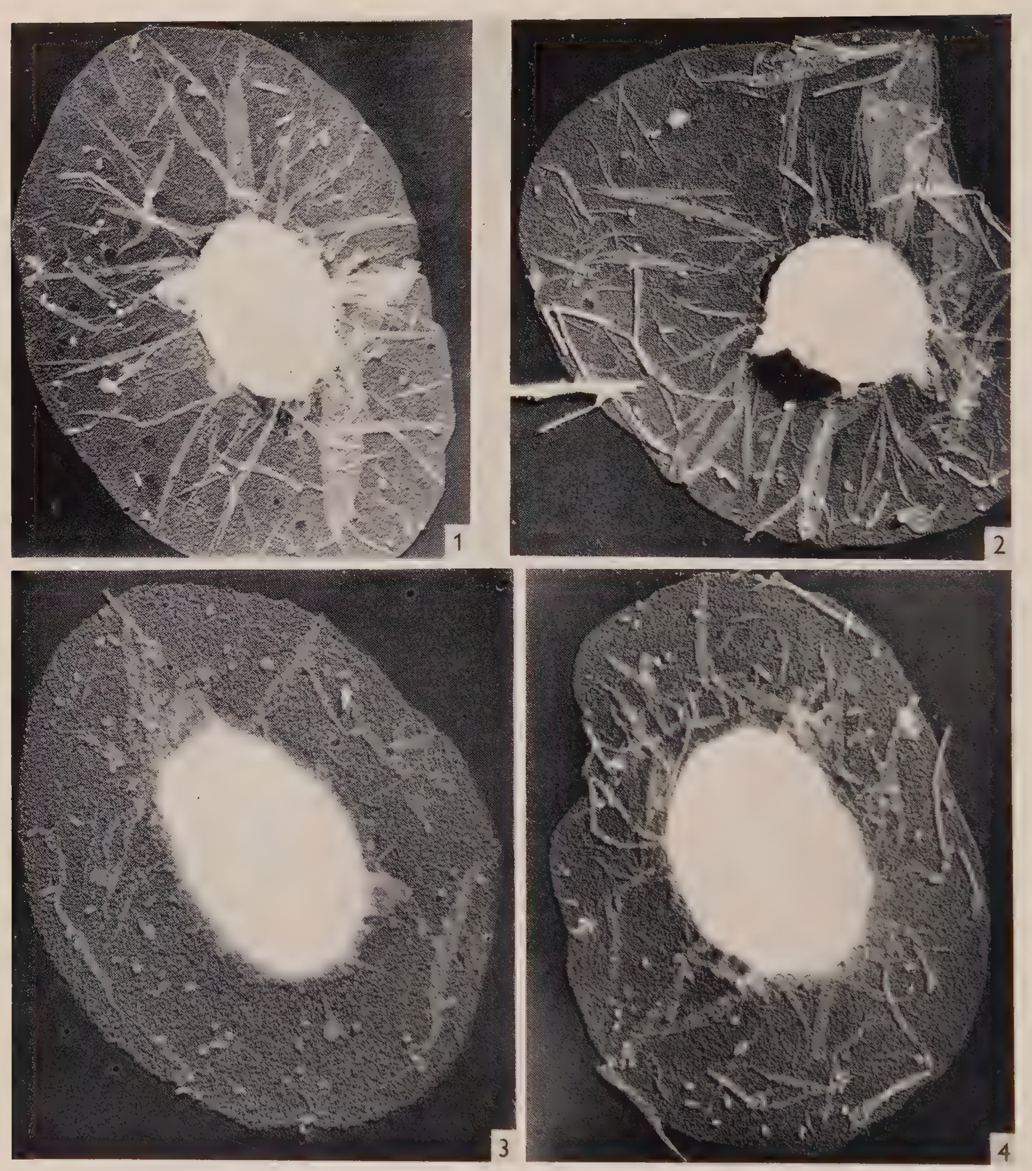

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EXPLANATION OF PLATE

Fig. 1. Influenza virus (A/Persian Gulf/2/52) adsorbed on a red cell ghost from egg treated with heated influenza virus, and showing a low proportion of filaments to spheres. Gold-manganin shadowed. × 14,000.

Fig. 2. Control. As fig. 1 but without pre-treatment of egg. There is a high proportion of filaments to spheres. Gold-manganin shadowed. × 14,000.

Fig. 3. Influenza virus (A/Persian Gulf/2/52) adsorbed on a red cell ghost from egg treated with metaperiodate, and showing a low proportion of filaments to spheres. Gold-manganin shadowed. × 12,000.

Fig. 4. Control. As fig. 3 but without pre-treatment of egg. There is a high proportion of filaments to spheres. Gold-manganin shadowed. × 14,000.

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Pigment Production by Staphylococci

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SUMMARY: White variants, which were stable over many generations, were isolated from six of eight strains of *Staphylococcus aureus* by cultivation in fluid medium; their incidence was favoured by 0·5% (w/v) lithium chloride. These variants all resembled the parent culture from which they were derived in the production of free coagulase and α-lysin and, except for one which was non-typable, in bacteriophage type. None of the white variants, however, was clumped by plasma in a slide test, although the parent cultures were.

Golden variants were isolated from two old laboratory strains of coagulase-positive staphylococci which had become white and from three of six white variant cultures isolated in the study of 'aureus→albus' variation. These golden variants were isolated under conditions which are unfavourable to the growth of staphylococci in the laboratory. They resembled the parents from which they were derived in production of free coagulase and α-lysin and in bacteriophage type. They differed from the parent cultures in production of the factor which causes clumping in plasma, and showed a non-specific increase in resistance to various antibacterial agents.

Although it has long been accepted that pigment production by staphylococci is not a reliable criterion of pathogenicity, nevertheless the majority of strains recently isolated from pathogenic processes do in fact produce a golden pigment. When such strains are repeatedly subcultured in the laboratory they tend to yield white variant colonies, and sometimes the whole culture becomes 'albus'. Chromogenic strains recently isolated from infective processes yield only occasional white variants in the usual laboratory media, but the number can be greatly increased by various means. Pinner & Voldrich (1932) found that when tuberculous pleural fluid, which causes agglutination of coagulase-positive staphylococci, or an antiserum prepared against a golden strain of staphylococcus was added to broth, eight to ten subcultures were sufficient to convert a golden culture to a pure white culture. Hoffstadt & Youmans (1932) found a high incidence of white colonies when *Staphylococcus aureus* strains were serially subcultured in broth containing 0·5% (w/v) lithium chloride.

White to gold variation is much less common and indeed only two records of such a change have been found. Pinner & Voldrich (1932) found that four white strains yielded golden colonies after 20 or more days in undiluted 'anti-albus' serum, although over a period of 2 years none had yielded golden colonies spontaneously. Byatt, Jann & Salle (1948) claimed that a cell-free extract prepared from a highly chromogenic strain of *Staphylococcus aureus* was capable of inducing chromogenesis in colonies of a white strain of the same species.

The present investigation was undertaken as a result of a chance observation made during other work. Two toxigenic strains of staphylococci were being maintained in mixed culture. Strain *a* appeared white and was penicillin-destroying and streptomycin-sensitive; strain *b* produced a deep golden pigment and was penicillin-sensitive and streptomycin-resistant. After serial subculture together at various intervals during many months the only variant colonies isolated had the antibiotic sensitivity of strain *a* but appeared to have the pigment producing capacity of strain *b*. The investigations of Byatt *et al.* (1948) appeared to offer a possible explanation for this result. These workers found that when a white strain of *Staphylococcus aureus* was grown in the presence of a cell-free extract of a golden strain of the same species, a proportion of golden colonies appeared. An extract of my strain *b* was therefore prepared according to the method of these workers and, again following their technique, my strain *a* was subcultured daily for 5 days in broth + extract of strain *b*; each day the broth cultures of strain *a* were plated on nutrient agar. The results obtained were similar to those of Byatt, *et al.* (1948); in repeated experiments different, but often very high, proportions of colonies were deeply pigmented. Since in the process of extraction chloroform, amyl alcohol and ethanol were used, strain *a* was also subcultured in broth containing small quantities of these substances, but without extract of strain *b*. When subcultures were carried out in the presence of chloroform or ethanol golden colonies appeared, and the incidence of these after subculture in the presence of chloroform was comparable to that obtained with extracts of the chromogenic strain. It seemed clear from these observations that Byatt *et al.* (1948) were unjustified in concluding that their results were due to a transforming principle extracted from a chromogenic strain, and a more detailed study of pigment variation in strains of staphylococci seemed desirable.

METHODS

'*Aureus* → *albus*' variation

Strains. Eight strains of *Staphylococcus aureus* yielding typical golden colonies on nutrient agar plates were selected; seven had been recently isolated; six (strains: W 54/36, 37, 38, 39, 40, Rex) from infective processes and one (W 54/129) from a nasal carrier; the remaining culture (V.P. 1) appeared to be a laboratory contaminant. All gave strongly positive reactions in coagulase and α-lysin tests, and all produced penicillinase except Rex which was penicillin-sensitive. When tested with bacteriophage, three strains (W 55/36, 40, 129) were found to belong to group I, one strain (V.P. 1) to group II, three strains (W 54/37, 38, Rex) to group III, and one strain (W 55/39) was non-typable.

Cultural procedures. The strains were serially subcultured in Lemco broth (1) alone at pH 7·4; (2) +0·5% (w/v) lithium chloride at pH 7·8 (as recommended by Hoffstadt & Youmans, 1932); (3) +4% (v/v) staphylococcal antitoxin (Burroughs Wellcome and Co.). At intervals the broth cultures were plated on Lemco agar and Chapman's (1946) glucose + mannitol + salt medium. These plates were examined after incubation for 18–24 hr. at 37° and again after several days at room temperature.

'*Albus → aureus*' *variation*

Strains. Most of the experiments were carried out with two old laboratory strains which in the course of time had ceased to yield golden colonies under the usual cultural conditions. One of these, strain D 3, is a penicillin-destroying organism of phage group III, isolated in 1948 from the air of a maternity department. The other, NCTC 6571 (Oxford staphylococcus), is a penicillin-sensitive organism of phage group I and has an even longer laboratory history. Both have retained their ability to clot plasma and to produce α-lysin, although strain NCTC 6571 clots plasma slowly and yields only traces of α-lysin. Neither is clumped by plasma. Experiments were also carried out with six of the white variants isolated in the study of 'aureus → albus' variation (37 LCalb, 37 Salb, 38 LCalb, 39 Bralb, 129 LCalb, V.P. 1 alb) and with three coagulase-negative strains kindly supplied by the late Professor F. R. Selbie (S 233, 237, 241).

Cultural procedures. Following the preliminary observations with broth containing small quantities of chloroform, various procedures were adopted which created conditions unfavourable for the growth of staphylococci. These included serial subculture in Lemco broth + 2–4 % (v/v) chloroform or staphylococcal antiserum; growth in the presence of sublethal concentrations of penicillin or streptomycin; 3–6 weeks incubation in nutrient broth. As in the study of 'aureus → albus' variation, the fluid cultures were plated out at intervals on Lemco agar and Chapman's medium.

Controls of technique. In all procedures involving serial subculture in fluid medium, uninoculated medium was similarly transferred to fresh tubes to test for the likelihood of contaminants. In over 100 such control subcultures one or two contaminating colonies were present on the plates in four instances, but in only one instance was the fluid medium contaminated, so that in the other three, further plating of the broth was negative. Variant colonies, whenever they appeared, were tested for coagulase production, α-haemolysin, sensitivity to penicillin and streptomycin and phage-type, to see whether they resembled the parent culture in these tests.

Bacteriophage typing. As a routine, variant cultures were tested only with four phages (3A, 3C, 47, 52A) since all the parent cultures were sensitive to one or other of these; the method used was that of Williams & Rippon (1952). The phage-types of a proportion of cultures were kindly confirmed by Dr R. E. O. Williams of the Central Public Health laboratory, who used the full range of phages.

RESULTS

'*Aureus → albus*' *variation*

Growth in broth. Although all strains yielded colonies which varied considerably in depth of pigmentation, white colonies were only isolated from two of the seven recently isolated strains (55/39, 129) and only occasionally from these. Strain V.P. 1 was much more unstable and yielded from 3 to 90 % of white colonies after various periods of subcultivation in broth, although such colonies were rare after surface cultivation on agar.

Growth in broth + lithium chloride. After 1 or more weeks, with or without subculture in broth containing 0·5% (w/v) lithium chloride, plate cultures showed colonies which varied very greatly in size and pigmentation. The size ranged from pin-point to 1 mm. or more in diameter and, while some colonies showed a deep golden pigment, some were biscuit-coloured, some white, and some semi-transparent. White colonies were isolated from five of the eight strains: four of the five (W 55/36, 37, 38, 129) yielded occasional white colonies after seven subcultures at 3-day intervals; and the fifth strain (V.P. 1) yielded 90% after three subcultures. Rough colonies of the type described by Hoffstadt & Youmans (1932) were not seen.

Growth in broth + antitoxin. Colonies showing moderate variations in size and pigmentation were seen after 1 or more weeks in broth + 4% (v/v) staphylococcal antitoxic serum, especially when not subcultured. One strain (W 55/37) yielded a few white colonies after 3 weeks.

The white variants. White variants were isolated from six of the eight chromogenic strains of *Staphylococcus aureus.* Plate cultures prepared from them consisted entirely of white colonies, and this remained so after two or three subcultures. These variants retained their ability to clot plasma and to produce α-lysin. None of them, however, was clumped by plasma in a slide test, although golden colonies from the same plates and the parent cultures gave positive slide reactions. The white variants isolated from strains W 55/37, 38, 39, 129, and V.P. 1 were of the same phage-type as the parent cultures from which they were derived. The white variant isolated from strain W 55/36 in lithium chloride broth was non-typable. In no instance was a white variant found to belong to a phage-group different from that of the parent culture.

'*Albus* → *aureus*' *variation*

Strain NCTC 6571. This white strain yielded pigmented variants more readily than any of the other cultures studied. In six experiments this strain was subcultured daily in broth alone or in broth + 2 to 4% (v/v) chloroform, and in four of these experiments golden colonies were obtained in increasing and often very large numbers from the chloroform-containing cultures. The proportions (%) of golden colonies on each day in a typical experiment were as follows:

Day ...	1	2	3	4	5	6	7
	% golden colonies						
In broth	0	0	0	0	0	0	0
In broth + chloroform	0	1	10	8	20	60	90

In two of these experiments pigmented colonies were obtained from the broth cultures, but in relatively small numbers. Transparent and semitransparent colonies were sometimes isolated from the chloroform-containing broths.

Strain NCTC 6571 was next serially subcultured on streptomycin ditch-plates and a streptomycin-resistant colony selected for further tests. This colony, although it gave a pure growth of white colonies on the first few subcultures, proved even more unstable than the parent streptomycin-sensitive culture. Pigmented colonies were obtained when very young (1 hr.) or very old (3 or more days), broth cultures were plated and, as with the parent strain, the frequency of occurrence of these colonies was greatly increased by serial subculture in the presence of chloroform.

Further, white streptomycin-resistant colonies selected from NCTC 6571 were rather more stable in relation to pigment, but all yielded golden colonies in the presence of sublethal concentrations of penicillin. This was best demonstrated by the gradient plate technique of Szybalski (1952). On gradient plates with 0·1 unit penicillin/ml. initially in the upper layer, a zone of pigmented colonies was seen just beyond the minimum concentration of penicillin which caused partial inhibition of growth (Pl. 1, fig. 1).

Strain D3. Studies were made of the parent culture of strain D3 which is penicillin-resistant and streptomycin-sensitive and also of variants which had the following reactions: (1) penicillin- and streptomycin-resistant; (2) penicillin- and streptomycin-sensitive; (3) penicillin-sensitive and streptomycin-resistant. These cultures were studied after growth in broth containing chloroform or staphylococcal antiserum, after prolonged incubation in broth, and on penicillin gradient plates. Golden colonies were only isolated from the original penicillin-resistant and streptomycin-sensitive culture and from this only occasionally. Twice golden colonies were isolated from 4- to 6-week-old broth cultures, and once they appeared in increasing number on serial subculture in chloroform broth. No golden colonies were isolated, however, in ten other experiments with chloroform. Transparent or semitransparent colonies were isolated from all of the four kinds of variant when serially subcultured in chloroform broth.

White variants. Golden colonies were isolated from three of the six white variant cultures studied (39 Bralb, 129 LCalb, V.P. 1 alb). In each case the pigmented colonies were obtained when broth cultures which had been incubated at 37° for 6 weeks were plated out.

Coagulase-negative strains. No golden colonies were isolated from these strains, although search was made for them after serial subculture in chloroform broth, after incubation in broth for many weeks and on penicillin gradient plates. Strain S 241, however, yielded a large number of transparent and semitransparent colonies; the incidence of these was greatly increased by subculture in chloroform broth (Pl. 1, fig. 2).

Golden variants. The golden variants showed a gross difference in pigmentation from the parent strains from which they were isoloted. When first isolated, plate cultures consisted entirely of golden colonies, but all of the variants tended to yield white colonies on further subculture. Streptomycin-resistant pigmented variants of NCTC 6571 were particularly unstable and golden colonies surrounded by satellite white colonies were sometimes seen.

All the pigmented variants showed the same pattern of antibiotic sensitivity as the parent from which they were derived, although those isolated from antibiotic-sensitive parent cultures were frequently able to grow in slightly higher concentrations of these antibiotics. Those who have studied the antibiotic sensitivity of staphylococci on solid media will be familiar with the frequent intense pigmentation which occurs just before the inhibitory concentration is reached. This phenomenon differs from that described above, in that on subculture of the deeply pigmented zone in the absence of antibiotic, colonies of the same pigmentation as the parent culture are obtained once more.

The golden cultures showed free coagulase and α-lysin production similar to that of the parents from which they were derived, and all here recorded were of the same phage-type as their parent cultures. Unlike the parent cultures most, but not quite all, of the golden variants were clumped by plasma in slide tests. In preliminary experiments golden colonies of phage-type 3 B/3 C/55/7 were twice isolated from strain D3 which is of phage-type 6/7/47/53/54/75+. These variants have been excluded from the analysis.

The golden variant colonies were frequently, but not always, smaller than the white colonies (cf. Pl. 1, fig. 3). When this was the case growth curves in broth showed that the pigmented culture had a longer lag phase than that of the parent, although after 24 hr. of incubation both cultures had multiplied to a similar degree.

Golden variation was associated with a slight but definite increase in resistance to various agents (e.g. chloroform, phenol, penicillin, streptomycin), whereas parent type cultures grew more readily in the usual laboratory conditions. This was best demonstrated by serial subculture of a mixture of organisms in the presence and absence of the antibacterial agent. Thus when an inoculum containing a proportion of one golden variant to 100 white (parent-type) organisms was used and serial transfer of the mixed culture made in broth or in broth+4% (v/v) chloroform, the proportion (%) of golden colonies was as follows:

Day ...	1	2	3
	% golden colonies		
In broth culture	<1	<1	0
In broth+chloroform culture	4	10	25

Thus the golden variants disappeared in broth, whereas they daily increased in numbers in broth+chloroform.

Some of the golden variant cultures survived heating to 60° for 1 hr., although the parent cultures did not survive this treatment. This property, however, was not limited to pigmented variants, as some white colonies isolated in the presence of chlorofrom or antibiotics showed a similar increase in resistance to heat.

DISCUSSION

It is well known that strains of *Staphylococcus aureus* when cultivated *in vitro* tend to lose their capacity to produce a golden pigment. This loss, which is usually first seen in only a proportion of the organisms of the culture, is sometimes associated with a diminished ability to produce α-haemolysin and to clot plasma. In the present investigation white colonies were only isolated occasionally from any of the seven recently isolated strains of *Staphylococcus aurues* and not at all from two of them. They were never isolated from surface cultures on agar, but only after subculture in fluid medium. Their isolation was favoured by the presence of 0·5% (w/v) lithium chloride (cf. Hoffstadt & Youmans, 1932).

The white variants appeared to resemble organisms of the parent culture in all other properties except that they were no longer clumped by plasma. It has been shown by Duthie (1954) that staphylococci produce two forms of coagulase, one of which is bound to the cell wall and causes clumping in plasma, while the other is free and clots the plasma. The loss by these strains of the clumping factor without loss of the power to clot plasma supports this view.

The 'albus → aureus' transformation is not a generally accepted phenomenon, although Pinner & Voldrich as long ago as 1932 described its occurrence when four white variants of golden strains were cultivated for long periods in undiluted homologous antiserum. The observations of Byatt *et al.* (1948) are also relevant although, as indicated above, their conclusions seem improbable in the light of the work recorded here.

It is clear from the present work that certain apparently white strains of coagulase-positive staphylococci may yield golden colonies under a variety of conditions which are unfavourable to growth. The most likely explanation of these results is that these golden variants are naturally occurring mutants which, since they show a relative increase in resistance to various antibacterial agents, tend to be selected by them. In this case white variants of golden cultures must also be mutants. That these are much more common is in accordance with current genetical views, since the forward mutation (or loss of a property) is usually much more frequent than the backward mutation (or gain of a property).

Hoffstadt & Youmans (1932) isolated eight 'rough forms' as well as small colony or G type variants from a single strain of *Staphylococcus aureus*. When this strain was cultivated in lithium chloride broth the following succession of changes was recorded: smooth yellow (aureus), smooth white, rough white, pseudo rough white, unstable G form, relatively stable G form, which, however, tended to revert to the smooth yellow or white form. Coagulase and haemolysin tests were not done, but only the smooth yellow cultures were virulent for rabbits. Similar dissociated forms were isolated from blood cultures of rabbits which had been inoculated with the same strain of *S. aureus*, but under these conditions G forms appeared more rapidly. The G forms passed through a Berkefeld filter and showed an increase in thermal death point from 55 to 75°. In their discussion the authors suggest that these dissociated forms are more in the nature of cyclostages than mutants or variants.

In the present work it was found that cultivation in lithium chloride broth led to marked variation in colony size and relatively stable tiny colony variants resembling G forms were sometimes isolated. Colonies resembling the rough forms of Hoffstadt & Youmans were not observed, although I have occasionally seen such colonies in old laboratory cultures of staphylococci.

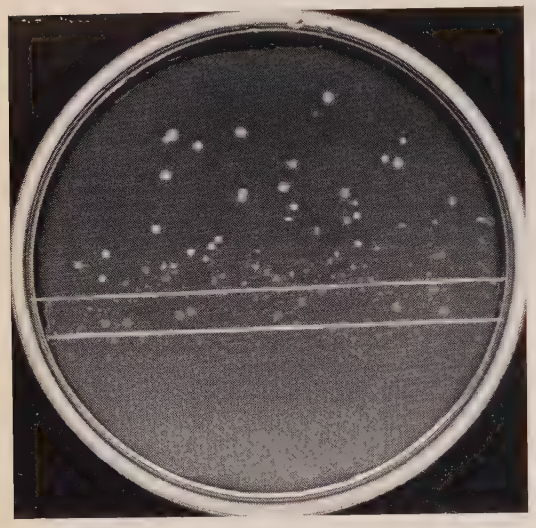

Fig. 1

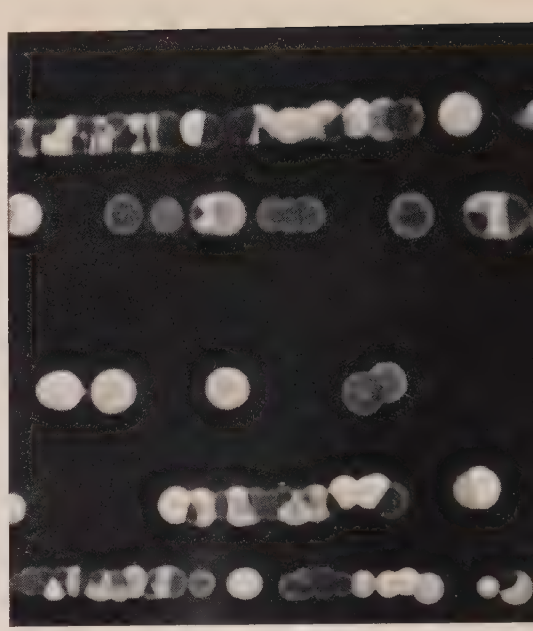

Fig. 2

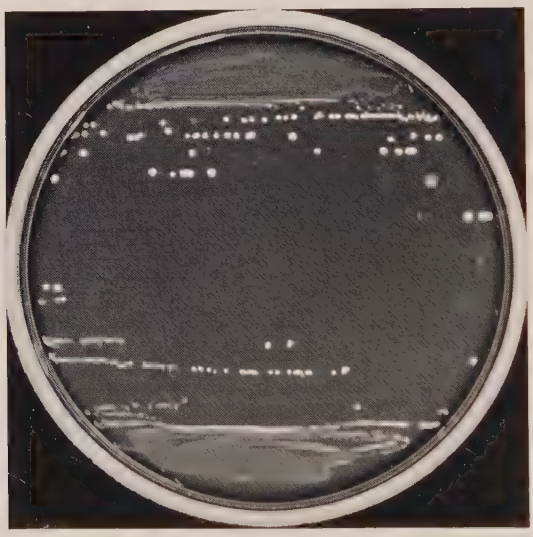

Fig. 3

M. Barber—Pigment production by staphylococci. Plate 1

(*Facing p.* 345

Since loss of production of a golden pigment is often associated with diminution or loss of other properties it is tempting to suggest that, under the usual laboratory conditions, a series of mutations takes place until a fully virulent strain of *Staphylococcus aureus* becomes an avirulent, non-toxigenic, white or even transparent staphylococcus. If this be the case, staphylococci should, perhaps, be regarded as a single species, of which the white coagulase-negative variety is but the laboratory end-product of golden coagulase-positive organisms. Pinner & Voldrich (1932) even suggested that the difference between 'aureus' and 'albus' cultures is of the same nature as the smooth to rough variation of other micro-organisms. In any case since pigment production is such a variable character it is doubtful whether this should be perpetuated as a basis for classification by use of the terms *Staphylococcus aureus* and *S. albus*.

My thanks are due to Dr R. E. O. Williams for confirming the bacteriophage type of many of the cultures, and to Mr A. L. Wooding for the photographs.

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EXPLANATION OF PLATE

Plate 1

Fig. 1. Penicillin gradient plate seeded with NCTC 6571 (Oxford staphylococcus). Large colonies between the two white lines were golden.

Fig. 2. Strain S 241 plated on nutrient agar after passage in chloroform broth showing large numbers of transparent colonies.

Fig. 3. Strain NCTC 6571 parent culture (upper half) and aureus variant (lower half) plated out on nutrient agar. Note smaller size of colonies of aureus variant.

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Recombination with Herpes Simplex Virus

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SUMMARY: The behaviour of six strains of herpes simplex virus was studied. Marked differences were noted in the type of lesion formed and the rate of attachment when the strains were inoculated on to the chorio-allantois, and in the behaviour of strains inoculated intracerebrally into mice or on to the cornea of the rabbit.

Two easily differentiated strains were inoculated together on to the chorio-allantois under conditions designed to give rise to a proportion of doubly infected cells. After a short period of incubation the progeny of the first intracellular growth cycle was obtained.

Ten clones were isolated, of which six resembled one of the two parents in the type of lesion formed and level of virulence for mice. Of the other four, one (106a) produced small type lesions and the other three large type. All possessed degrees of virulence intermediate between those of the parents. Three of these substrains showed no alteration in the type of lesions formed or their degrees of virulence after 3 to 5 passages of single lesions at limiting dilution. When the rate of attachment to the chorio-allantois and the behaviour when inoculated on to the cornea of the rabbit were studied, it was found that 106a produced effects intermediate between the parents, whilst the other strains resembled one of the two parents.

Genetic recombination has now been demonstrated amongst several viruses. Delbrück & Bailey (1946) and Hershey & Rotman (1948) showed genetic interaction with bacteriophage. More recently, conclusive evidence of recombination has been provided amongst the influenza viruses (Burnet & Lind, 1951; Burnet, Fraser & Lind, 1953; Hirst & Gottlieb, 1953; Perry, van den Ende & Burnet, 1954). No animal virus other than influenza has yet been shown to exhibit this type of genetic interaction. The object of the present work was to discover whether herpes simplex virus would recombine when subjected to conditions like those required for recombination in other viruses. Before this was attempted, a study was made of the characteristics of six strains of herpes virus in order to find suitable markers by which to identify the progeny.

MATERIALS AND METHODS

Virus strains. Six strains of herpes simplex virus were used. One strain (Eri) was recently isolated from a case of labial herpes and was used after six passages on the chorio-allantois. The other five have been maintained in the stocks of the Walter and Eliza Hall Institute for some years. The strain H.F.E., which had been adapted to grow on the chorio-allantois by Burnet & Lush (1939*a*) originated from the Rockefeller Institute strain H.F. The mouse-adapted strain H.F.E.M. had been adapted from strain H.F.E. and is not directly descended from the mouse-adapted strain described by Burnet & Lush

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(1939*b*). The remaining strains Nag, Sch and W.C. have been isolated over the years at this Institute.

Maintenance of strains. The strain H.F.E.M. had been maintained by intracerebral passage in mice; all other strains had been maintained by chorio-allantoic passage. Suspensions of virus used were first obtained as pure clones by the method outlined below. All strains were then passaged once on the chorio-allantois from which suspensions were made by grinding with alundum. These suspensions were stored at $-70°$ in ampoules.

Diluting fluid. All dilutions of virus were made in broth (Wright, 1933) to which was added streptomycin and penicillin to give respectively 200 μg./ml. and 14 units/ml.

Egg techniques. The conventional egg techniques used were those described by Beveridge & Burnet (1946). All titrations of infectivity for eggs were made with serial tenfold dilutions of virus and inoculating each on to the chorio-allantois of four eggs at the 11th or 12th days of incubation. The infective unit (i.u.) is defined as the quantity of virus necessary to give rise to one lesion on the chorio-allantois after incubation at 35° for 48 hr.

The rate of attachment of virus to the chorio-allantois was measured as previously described (Wildy, 1954). Briefly, 0·05 ml. virus suspension was inoculated on to the chorio-allantois of a number of eggs at a dilution which gave 50 to 100 lesions/membrane. After the required period, the proportion of virus remaining unattached was determined in eight of the eggs. To each egg, 0·1 ml. broth was added and the hole in the shell obliterated with paraffin wax. The egg was then rotated about its long axis through 120°. This manoeuvre drives the bubble of air in the artificial airspace to a new position, and the fluid containing unattached virus is carried to a new area on the chorio-allantois. After incubation at 35° for a further 48 hr. the membranes were removed intact and the lesions counted on both the first and second areas.

Isolation of pure clones. It has been assumed that any specific lesion formed on the chorio-allantois is caused by one virus particle. On this assumption, the validity of which will be discussed later, pure clones were isolated by grinding lesions formed at limit dilution, that is where lesions occurred singly per membrane.

Specific neutralization. Herpes neutralization tests were carried out using the method of Burnet & Lush (1939*a*). For this purpose and for removing unwanted extracellular virus in the recombination experiment, the serum used was derived from rabbits which had been immunized against strain H.F.E. (Wildy, 1954).

Animal techniques. Mice of the Walter and Eliza Hall Institute stock strain were used during the fourth week of life. Titrations of virus were made by inoculating tenfold dilutions of virus suspensions each into four mice. Each mouse received 0·05 ml. virus intracerebrally. The mice were observed over a period of 14 days and all deaths in mice showing signs of encephalitis were regarded as specific. The end-points taken were at the dilution of virus required to give 50% mortality (LD 50), which was calculated by the method of Reed & Muench (1938).

The degree of virulence for mice was measured by making parallel titrations of suspensions of virus in mice and in eggs, using the same set of dilutions for each. From the values obtained, the degrees of mouse virulence have been expressed quantitatively as the *mouse virulence ratio* (m.v.r.). This value is defined as the number of infective units required to constitute one 50 % lethal dose for the mouse under the conditions of the test (i.e. m.v.r. = i.u./LD 50). It may be noted that this value varies inversely with the virulence of the strain for mice.

Rabbits were inoculated as young adults by scarification of the cornea whilst under ether anaesthesia, followed by the instillation of 0·05 ml. virus suspension containing from 10^4 to 10^5 i.u. They were then observed for a period of 14 days.

RESULTS

Characterization of six strains of herpes simplex virus

In order to find two strains with readily and clearly defined marker characteristics, a study was made of the behaviour of six strains of herpes simplex virus, the relevant features of which are recorded in Table 1.

Serological uniformity. All the strains were tested for specific neutralization by the method of Burnet & Lush (1939*a*). In every case, the serum which had been prepared against H.F.E. neutralized the virus.

Preliminary experiments had shown that the six strains behave differently in eggs. The character of the lesion produced, the rate of increase of infective virus and the rate of attachment to the chorio-allantois all differed from strain to strain.

Character of the lesions. The character of the lesions formed was investigated by inoculating a number of eggs each with 50 to 100 i.u. of the strain in question and incubating at 35°. At daily intervals, four eggs were taken and the lesions carefully examined. It was found that the four strains H.F.E.M., H.F.E., Sch and Eri caused large opaque lesions (LO lesions) and these are referred to subsequently as LOL strains. These lesions generally measured 1·0–1·5 mm. diameter, but the size varied from egg to egg and to some extent inversely with the number of lesions on the membrane. The other two strains Nag and W.C. gave small lesions (S lesions) which never exceeded 0·5 mm. diameter. These are subsequently referred to as SL strains. With the LO lesions there was a tendency for the centres of a proportion of them to become necrotic and sometimes haemorrhagic. This was common after incubation for 48 hr. and constant after 72 hr.—S lesions, on the other hand, never became necrotic, even after incubation for 5 days. In fact it was always possible to tell at once with which type one was dealing. Membranes showing typical lesions are depicted in Pl. 1.

Extent of multiplication in 48 hr. The extent of multiplication in the chorio-allantois was estimated by inoculating 50–100 i.u. on to the chorion and harvesting the membranes after 48 hr., but because the membranes were ground with alundum, the results are regarded as being very approximate (cf. Anderson, 1954). It was, however, obvious that the LOL strains gave increases which were more than 100 times those of the SL strains.

Table 1. *Summary of characteristics of six strains of herpes simplex virus*

Mouse virulence ratio (m.v.r.) given $\log_{10}$. Neutralization test carried out by the method of Burnet & Lush (1939*a*).

Strain	Neutralization (%)	Proportion unattached to chorio-allantois 30 min. after inoculation (%)	Lesion		Extent of multiplication (48 hr.)	No. tests	m.v.r. (mean)	No. tests	Rabbits	
			Size (48 hr.)	Necrosis (120 hr.)					Keratitis	Encephalitis
H.F.E.M.	0	22 (19+25)	LO	+	×6·5 $\log_{10}$	4	0·14 ($\bar{1}$·73–0·5)	2	+	+
H.F.E.	2	31 (29+33)	LO	+	×4·8 $\log_{10}$	3	1·43 (0·86–1·7)	3	+	+
Sch	3	22	LO	+	×6·0 $\log_{10}$	1	1·57	1	+	+
Eri	4	28	LO	+	×5·8 $\log_{10}$	1	2·05	1	+	+
Nag	2	27	S	–	×3·7 $\log_{10}$	2	>4·42 (>4·42– >4·26)	2	+	–
W.C.	4	51·5 (49+52)	S	–	×3·0 $\log_{10}$	4	>5·1 (>4·08– >5·1)	3	–	–

Rate of attachment to the chorio-allantois. The proportion of virus remaining unattached was determined 30, 60 and 120 min. after inoculation. It was found that there was little difference between the strains H.F.E.M., H.F.E., Sch, Eri and Nag, where the proportion of virus remaining free was at 30, 60 and 120 min., respectively, 22–31, 7–13 and 4–10 %. On the other hand, W.C. became attached much more slowly, the equivalent values being 50·5, 30 and 20 %. Only the 30 min. values are given in Table 1.

Virulence for mice. The virulence for mice was determined as described above. With strains H.F.E.M., H.F.E., Nag and W.C. separate determinations were made with suspensions of virus derived from successive passages on the chorio-allantois. It may be seen in Table 1 that the LOL strains all gave rise to encephalitis and death in mice, whilst the SL strains (Nag, W.C.) never showed this potentiality in the concentrations tested. Moreover, the degrees of virulence of the LOL strains are distinct, decreasing in the order given. It is also apparent that the strains which were tested upon more than one occasion gave a range of m.v.r. of a little less than a $\log_{10}$ unit.

Behaviour in rabbits. Of a number of animal species tested for susceptibility to infection, clear-cut differences were only observed with the rabbit when inoculated on the cornea. The results were as follows. All the strains except W.C. produced visible keratitis in the inoculated eye within 2–4 days. Of these, all except strain Nag gave severe conjunctivitis, blepharitis and encephalitis and death within 7–11 days of inoculation. It is of interest that Nag gave rise only to keratitis whilst the other SL strain, W.C., gave rise to no visible abnormality.

Table 1 summarizes the main characteristics of the six strains studied. Differences of a similar sort have been reported previously by several authors (Burnet & Lush, 1939*b*; Florman & Trader, 1947; Kilbourne & Horsfall, 1951). It is of interest that all the LOL strains produced encephalitis either in rabbit or in mouse. These LOL strains all multiplied to a similar extent within 48 hr. and all were capable of producing lesions with a central necrotic crater. The SL strains, on the other hand, did not produce encephalitis, multiplied more slowly and never produced necrotic lesions.

When the rate of attachment to the chorio-allantois and the production of keratitis in the rabbit are considered, the demarcation comes in a different place. The LOL strains and Nag all give keratitis and become attached rapidly; strain W.C. which gives no clinical keratitis and become attached more slowly.

It has been possible to measure the virulence for mice of the L strains on a quantitative basis. When the results of this test are combined with the foregoing results, it is possible to arrange the six strains in a definite order of 'activity' as they are given in Table 1.

General plan of the recombination experiment

Of the six strains which had been studied, H.F.E.M. and W.C. were chosen as prospective parents. Although this choice introduced difficulties which will become apparent below, it did make use of the strains available which were the most widely different. Before recombination was attempted, two essentials had to be satisfied: (1) the ratio infective virus/susceptible cells had to be great enough to ensure that a proportion of susceptible host cells were doubly infected with both parents; (2) a method had to be devised for obtaining virus from the first intracellular growth cycle which was not contaminated with unincorporated virus.

Multiple infection of cells.

Of the tissues available in which herpes virus will grow, the chorio-allantois is probably the simplest to use. It possesses the advantages that infection may be restricted to a small area of its surface and that reagents may be placed upon it with ease. Tyrrell, Tamm, Forssman & Horsfall (1954) estimate that the total number of cells in the allantois is about $10^{7\cdot25}$. But for our purpose it is better to use the higher estimate of $10^{8\cdot3}$ made by Cairns, Fazekas de St Groth & Edney (1952). It has been assumed that the number of cells in the entire chorion is of a similar order, but because only about one-eighth of the total chorion is exposed to infection, it is safe to assume that the cell content of the infected portion is about $10^{7\cdot25}$. Thus more than 10^8 i.u. virus should suffice to ensure multiple infection of a proportion of cells.

It was found possible to obtain suspensions of strain H.F.E.M. which contained adequate concentrations of virus by inoculating into the yolk sacs of eggs at the eighth day of incubation and harvesting the amniotic fluids after a period of further incubation at 35° of 4 days (Nagler, 1946). With strain W.C., on the other hand, it was not found possible to obtain suitable concentrations of virus by this means alone. Adequate concentrations were, however, obtained by collecting the amniotic fluids from 100 eggs which had been inoculated in this way and centrifuging for 60 min. at 12,000 g. on a Spinco preparative ultracentrifuge.

Method of obtaining the progeny of the first intracellular growth cycle free from contamination from unincorporated virus

Preliminary experiments with strains H.F.E.M. and W.C. revealed that with each, when a large inoculum was used, new mature virus could be obtained at 7 hr. after inoculation. But, because of the differences in yields and rates of attachment of the two strains, it was decided to give strain W.C. an advantage by inoculating it 1 hr. before strain H.F.E.M. and to harvest the membranes 7 hr. after the second inoculation.

When harvesting the membrane at 7 hr., there is danger of including with the newly matured virus a part of the original inoculum which had remained extracellular. Because both strain H.F.E.M. and strain W.C. were neutralized by the same antiherpes serum, it was decided to bathe the surface of the chorion with this serum after allowing sufficient time for the strains to penetrate the chorionic cells.

Details of the recombination experiment

The chorio-allantoic membranes of two eggs at the 11th day of incubation were dropped, using 0·05 ml. broth. Then 0·1 ml. of a suspension of strain W.C. containing 10^8 i.u. was placed on the membrane and after incubation at 35°

for 1 hr., 0·05 ml. of a suspension of strain H.F.E.M. containing $10^{8\cdot4}$ i.u. was added. After incubation for 1 hr. more, 0·5 ml. specific neutralizing serum was placed on the membrane and the eggs again incubated for another 6 hr. The membranes were then removed and washed thrice in sterile saline, pooled and homogenized with alundum and 1·0 ml. broth. The material was centrifuged at 2000 r.p.m. and the supernatant fluid collected. In parallel, control eggs were inoculated separately with either parent. These eggs were then treated in the same way as those which were doubly infected.

Isolation and characterization of clones of progeny

It had been decided to isolate ten clones of the progeny of the mixed infection and five each from the two single infections, then these twenty clones isolated were to be characterized and tested for stability. Such an undertaking would have required large numbers of animals and eggs and have taken a long time. It was, therefore, decided to screen each of the clones on the basis of two characters only, and to investigate fully only those clones whose behaviour differed from the parents.

Isolation of clones. The suspensions obtained from the mixed infection and the two controls were titrated for their content of infective virus. Where fewer than five lesions occurred on a membrane, the lesions were carefully removed and ground separately with a small amount of alundum and 1·0 ml. broth. It is recognized that at this stage there was the opportunity for a small amount of virus from other lesions on the same membrane to contaminate the clones, but, it is believed, not sufficient to affect the results of the screening test. Subsequent manoeuvres are assumed to have purified the clones.

Screening test. In order to avoid unnecessary wastage, the screening of clones was carried out in two parts. All strains were first subjected to a short test and subsequently, those which gave unexpected or doubtful results were tested fully by the method of parallel titration.

Short test. Whilst determining mouse virulence ratios, it was noted that the number of i.u. required to kill 50 % of the mice on a particular day after inoculation was fairly characteristic of a strain. If, therefore, the approximate concentration of infective units in a suspension is known, the time that elapses between its inoculation into mice and 50 % of deaths provides a fair indication of mouse virulence. Thus the twenty suspensions obtained from the recombination experiment and controls were each inoculated undiluted into the brains of four to six mice and on to the chorio-allantois of four eggs at a dilution of 10^{-1}. When the eggs were examined after incubation for 48 hr., the lesions were counted and their type noted. The times at which the mice died were also noted. The results are summarized in Table 2. All suspensions derived from strain H.F.E.M. produced LO lesions and all killed mice. Four of those derived from strain W.C. gave S lesions and one gave no lesions at all on the egg. None killed mice. Of the suspensions derived from the mixed infection, one (106 a) gave S lesions and the remainder LO lesions. Only the latter killed mice, but it will be noted that the time taken to kill 50 % was variable.

Table 2. *The behaviour of progeny of mixed infections and controls in the screening test and, in some instances, after one passage on the chorio-allantois*

Strain	Clone no.	Lesion	Screening test			After one passage on the chorio-allantois	
			i.u. inoculated ($\log_{10}$)	Days elapsed to 50 % death		Lesion	m.v.r. ($\log_{10}$)
W.C.	125	S	1·45	—	.	.	.
	129 a	S	1·25	—	.	.	.
	129 c	S	2·24	—	Passaged	S	> 3·93
	133	S	1·90	—	.	.	.
H.F.E.M.	077	LO	2·64	5·25	.	.	.
	079	LO	3·10	4·5	.	.	.
	081	LO	3·46	3·0	.	.	.
	083 a	LO	2·87	5·0	.	.	.
	083 b	LO	3·13	5·5	Passaged	LO	0·13
Mixture	106 a	S	2·04	—	Passaged	S	4·03 (3·9 + 4·13)
	106 b	LO	2·91	5·0	Passaged	LO	0·18
	106 c	LO	3·20	9·0	Passaged	LO	2·22
	107	LO	3·28	3·4	.	.	.
	108	LO	2·77	3·75	.	.	.
	109	LO	3·02	3·25	.	.	.
	110 a	LO	3·17	2·5	.	.	.
	110 b	LO	2·08	8·5	Passaged	LO	1·02
	110 c	LO	2·68	3·75	.	.	.
	110 d	LO	2·26	7·5	Passaged	LO	1·20

In order to evaluate these results, it is necessary to compare them with the relevant standards. In Fig. 1, the numbers of i.u. of strain H.F.E.M. which give 50 % of deaths at different times are plotted. The points (black dots) were derived from five separate parallel titrations and fall on a characteristic curve. It is not possible to construct a similar curve for strain W.C., but it is known that all values must be greater than 5·1 $\log_{10}$ i.u.

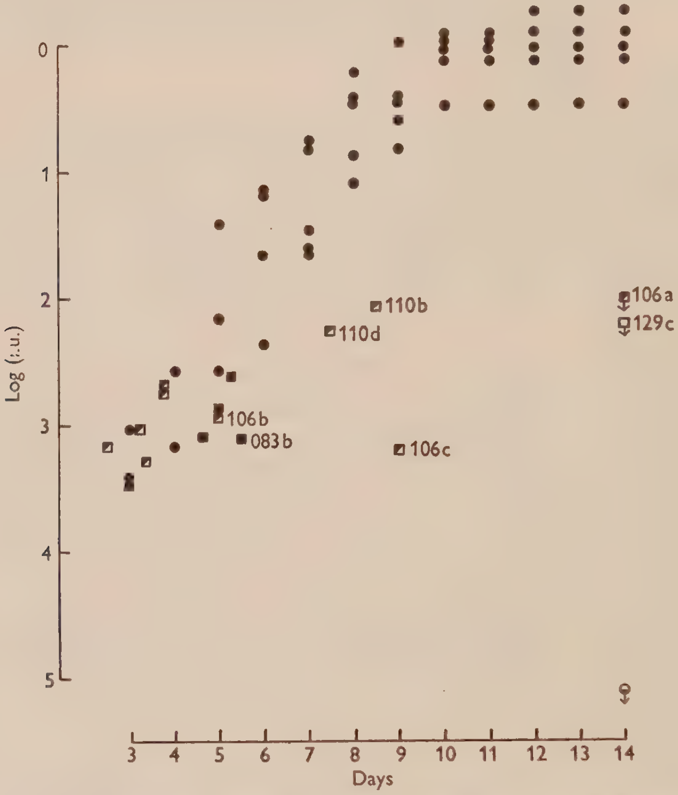

Fig. 1. The number of infective units required to kill 50 % of mice inoculated on particular days for strain H.F.E.M. (●). Superimposed are the results obtained in the screening of progeny of mixed infection ◩ and parents subjected to similar conditions (H.F.E.M., ■; WC, □). No mice were killed by strain W.C. (◒) in any experiment.

The values given in Table 2 have also been plotted in Fig. 1 (squares). It is to be expected that those which fall on the characteristic curve possess m.v.r. of the order of strains H.F.E.M., whilst those falling below and to the right of the curve possess higher m.v.r. values. In fact, it is obvious that with the exception of the borderline case 083b, the clones derived from the strain H.F.E.M. control fall within the H.F.E.M. curve. Of the ten clones derived from the mixed infection, six fall on this curve and four (106a, 106c, 110b and 110d) do not. The above four clones with three others (106b, H.F.E.M. 083b, and W.C. 129c) were passaged, once on the chorio-allantois, and the resulting suspensions tested by parallel titration.

Determination of lesion type and m.v.r. after one passage

The results obtained with the first passage suspensions are summarized on the right of Table 2. In all cases the types of lesion formed were the same as were noted in the short test. The clones H.F.E.M. 083b and mixed infection 106b both gave m.v.r. values of the order previously found with H.F.E.M. and, as expected, the clones 106c, 110b and 110d gave values which fell between those of the two parents. For the first time, 106a was inoculated in a sufficiently high concentration to kill mice so that this strain which gives S type lesions also possesses an m.v.r. value between the parents. It is unfortunate that the only clone of strain W.C. (129c) which was tested had a low concentration of infective virus, but it is significant that whilst this and 106a were inoculated into mice at about the same concentration, mice were only killed by the latter.

Stability of characteristics amongst clones from the mixed infection

If the alterations in virulence observed are to be ascribed to genetic changes, it is necessary to show that the quality is stable and the characteristic of a pure clone. Accordingly, the clones 106a, 106c, and 110b were passaged serially on the chorio-allantois. This was performed by the method for isolating pure clones where lesions occurring single on membranes were picked for passage. Between three and five passages were made in this way. During the process no alteration occurred in the type of lesions formed. Suspensions obtained from the final passage were titrated in parallel on eggs and in mice.

The mouse virulence ratios obtained are set out in Table 3. It is clear that single lesion passage preserved the essential characteristics of the clones. There was no alteration in the type of lesion formed, and 106a and 110b obviously retained their characteristic m.v.r. The clone 106c did show a fall in m.v.r., but as this was less than 1 log unit, it is interpreted as being within the range expected with this test (i.e. 1 log unit).

In view of the foregoing results, it was considered worth while to determine the ratio of attachment of the three clones 106a, 106c and 110b and to examine

Table 3. *The stability of the character of the lesions produced and m.v.r. given by three substances from the mixed infection after a number of single lesion passages on the chorio-allantois*

	First passage material		Subsequent single lesion passages (serial numbers of eggs)	Final passage material	
Substrain	Lesion	m.v.r. ($\log_{10}$)		Lesion	m.v.r. ($\log_{10}$)
106a	S	4·03	069→618→066→454→752	S	3·63
			↘067→456→748	S	4·02
			↘457→750	S	3·49
106c	LO	2·22	073→626→464→758	LO	1·79
			↘461→754	LO	1·26
			↘463→756	LO	1·35
110b	LO	1·02	087→472→760	LO	1·45

their behaviour in rabbits. For both purposes, the suspensions of virus used were obtained from the final single lesion passage.

Rate of attachment to the chorio-allantois. The rates of attachment were determined as before, but only after periods of 30 and 120 min. On the whole, the results obtained were clear. The three suspensions of 106a showed that the proportions of unattached virus at 30 min. were 38, 34 and 38 % and at 120 min. 11, 11 and 12 %. Those of 106c were 25, 37 and 25 % at 30 min. and 6, 6 and 14 % at 120 min., whilst that of 110b gave the values 27 % at 30 min. and 11 % at 120 min. It will be seen that while the three suspensions of 106a gave good agreement, there were two anomalous results with 106c. It has, unfortunately, not been possible to repeat the determinations, but it is considered that the values 37 % after 30 min. and 14 % after 120 min. are at fault. They were obtained with different suspensions (754 and 756) and appear incompatible with all other results with this type of experiment, and have, therefore, been disregarded. A summary of the results is given in Table 4; where they are compared with those previously obtained with the parents. It is clear that of all strains W.C. became attached most slowly to the chorio-allantoic membrane, and the results suggest that 106a became attached more slowly than 106c, 110b and H.F.E.M., all of which became attached at much the same rate.

Table 4. *The characteristics of parent strains W.C. and H.F.E.M. and three substances of progeny after single pock passage*

Parent strain	Lesion	Proportion unattached to chorio-allantois 30 min. after inoculation (%)	m.v.r. ($\log_{10}$)	Behaviour in rabbits K	Behaviour in rabbits E
W.C.	S	50·5	<5·1	−	−
H.F.E.M.	LO	22	0·14	+	+
Progeny					
106a (752)	S	38	3·63	+	−
(748)	S	34	4·02	+	−
(750)	S	38	3·49	+	−
106c (758)	LO	25	1·79	+	+
(754)	LO	37	1·26	+	+
(756)	LO	25	1·35	+	+
110b (760)	LO	27	1·45	+	+

K = keratitis E = encephalitis

Behaviour in rabbits. Each of the suspensions was inoculated as previously described, on to the cornea of one rabbit. The results (Table 4) were so clear-cut that it appeared unnecessary to use more animals. It is of interest that whilst 106c and 110b both gave keratitis, encephalitis and death, 106a only produced keratitis which ultimately disappeared. This behaviour is intermediate between the two parent strains.

Summary of results

The two strains H.F.E.M. and W.C. were inoculated together in such a way that double infection of a proportion of host cells was expected. Characterization of ten clones of the progeny revealed that four possessed types of lesion like one or other parent and levels of virulence for mice which were intermediate between those of the parents. With three clones, these characteristics were shown to be stable for three to five single lesion passages. The behaviour of suspensions derived from the last passage is summarized in Table 4, where the results previously obtained with H.F.E.M. and W.C. are also shown for comparison.

DISCUSSION

Five examples of polygenic inheritance of virulence amongst animal viruses have now been recorded (Burnet & Lind, 1954; Burnet, 1954). The evidence in favour of this hypothesis rests mainly on the results of recombination experiments where the progeny exhibits a wide distribution in the levels of virulence of individual clones intermediate between those of the parent strains.

The results obtained here suggest a similar phenomenon, but before they can be ascribed to genetic interaction, one must have reasonable evidence that certain assumptions are correct. First, the marker characteristics used must be clear-cut and stable. Secondly, the method of isolating pure clones must be certain. Thirdly, there should be evidence that the conditions imposed by the recombination experiment do not modify either parent when inoculated individually and lastly, pure clones of progeny isolated should remain stable on passage.

Of the marker characteristics employed here, it is clear that the level of virulence for mice, the type of lesion formed and the behaviour of the strains in rabbits all provide clear-cut results. The rate of attachment to the chorio-allantois which showed wide differences between the parent strains gave less clear-cut differences amongst the progeny.

Isolation of pure clones by the limiting dilution technique has been discussed fully by Burnet (Burnet & Lind, 1951). With herpes virus, an advantage is offered in that this virus produces easily visible lesions on the chorio-allantois. It has been assumed that each lesion is caused by only one virus particle. Three circumstances support this view. First, despite contrary reports (Rose, 1952), there exists, within limits, an approximately linear relationship between the dilution of virus suspension inoculated and the number of lesions resulting on the chorio-allantois (Beveridge & Burnet, 1946). Following the arguments advanced by Dulbecco (1952), this provides good evidence that each lesion is caused by one virus particle. A second hint is provided by the fact that with H.F.E.M., H.F.E., Nag and W.C. one egg infective unit is just sufficient to infect a mouse intracerebrally when the criteria for infection are taken as either death from encephalitis or ability of the inoculated mouse to survive intracerebral challenge with 100 i.u. H.F.E.M. 14 days after inoculation (Wildy, unpublished). This suggests that the infective doses for eggs and mice are the same, and the simplest interpretation is that in each case one virus particle is involved. The third circumstance is provided by analogy with the findings with vaccinia virus (Parker, Bronson & Green, 1941), which strongly suggested that each lesion was initiated by one virus particle. Although, therefore, there exists no unequivocal evidence that the lesions of herpes simplex on the chorio-allantois are initiated by a single virus particle, it is considered a reasonable assumption that this is so.

It is considered most unlikely that the conditions imposed upon parent strains during the recombination experiment would in any way modify their behaviour. It has unfortunately not been possible to characterize fully the ten clones of virus isolated from the control eggs in which parent strains were separately inoculated. It is clear, however, that in every case each parent strain gave rise only to its characteristic type of lesion and that on the basis of the screening test, the five clones of H.F.E.M. behaved typically with regard to their mouse virulence. The mouse virulence of W.C. has not been satisfactorily checked.

It is clear that the progeny of the mixed infection possessed stable characteristics. When the strains were subjected to serial single lesion passage, the types of lesion produced remained stable for each strain. Only in one case (106c) was there any shift in the m.v.r. (see Table 3) and this was commensurate with the variation in the values obtained previously with this test for H.F.E.M. and H.F.E. (cf. Table 1).

Quantitative experiment with animal viruses necessarily lacks precision. With herpes virus, which lacks the *in vitro* techniques applicable to influenza viruses, there exists no simple method of assessing the concentration of elementary bodies in a suspension. Hence, the expression of mouse virulence depends upon the comparison of two biological functions which have been expressed as the m.v.r. This value as an expression of mouse virulence obviously depends upon the egg infective unit remaining constant. Again, there is no unequivocal evidence that this is so, but the general experience of consistency with chorio-allantoic titrations suggests that it is the case.

The interpretation of the results in terms of genetic recombination is based upon the fact that clones isolated from the first intracellular growth cycle of a mixed infection exhibited stable characteristics which were different from either parent.

Too few isolations were made to infer any particular genetic patterns in herpes simplex virus, but the fact that recombinants were isolated with different degrees of virulence from the parent strains suggests strongly polygenic control of this quality. At the present time, very little is known about the genetic processes occurring in viruses like herpes simplex. It may be significant that the naturally occurring and recombinant strains which have been tested may be arranged so as to form an 'activity gradient' with the strain H.F.E.M. as the most active in all respects and strain W.C. as the least. It may well be that the differences with which we are concerned involve a simple factor 'activity' which depends in some way upon 'dosage' or number of one genetic determinant rather than upon several different genes.

Once the results have been accepted as genetic recombination, it follows that herpes virus resembles influenza virus in its mode of multiplication in that the genome becomes segregated in the early stages of growth. Evidence for the existence of an eclipse phase during the growth of herpes has already been provided (Scott, Coriell, Blank & Gray, 1953; Wildy, 1954). The inference that segregation occurs during the eclipse phase is obvious, but as there exists no evidence to this effect, this remains a probability only. The study on the appearance of soluble complement-fixing antigen during the first intracellular growth cycle of herpes (Wildy & Holden, 1954) suggested that this occurs with maturation of new virus rather than before, so that this is not thought to be related to the process of segregation and recombination.

It is a pleasure to acknowledge the interest and advice given by Sir Macfarlane Burnet who originally suggested the method of approach used in this work. Grateful acknowledgement is also offered to Miss Lois Larkin without whose technical competence the work could not have been completed. Thanks are also due to Mr G. L. Ada for concentrating suspensions of virus, to Mr Wooding for the photographs and to Miss Lester who drew the figure.

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EXPLANATION OF PLATE

Chorio-allantoic membranes of eggs inoculated at the 11th day of incubation after a further period of incubation at 35° for 48 hr: fig. 1, H.F.E.M., fig. 2, W.C. both magnified, ×2.

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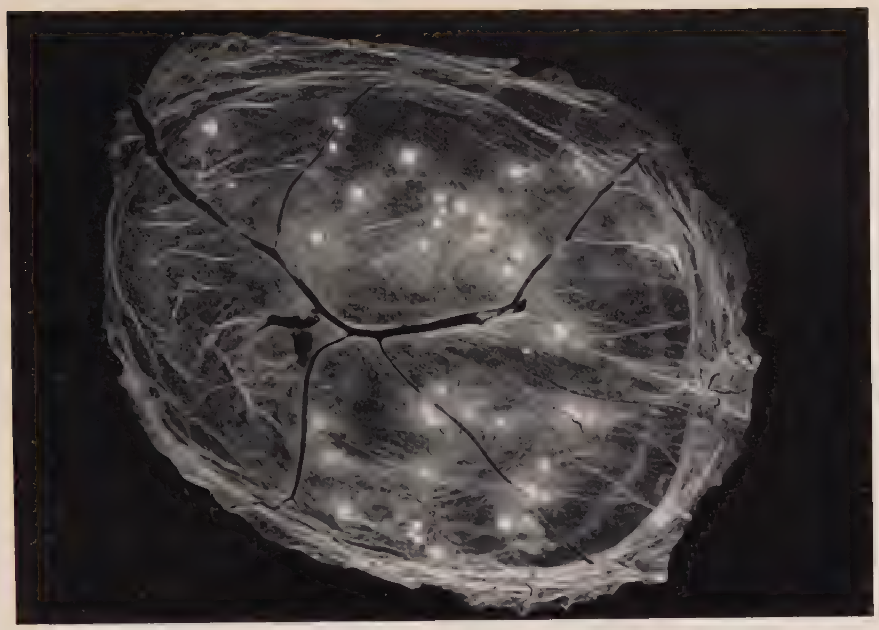

Fig. 1

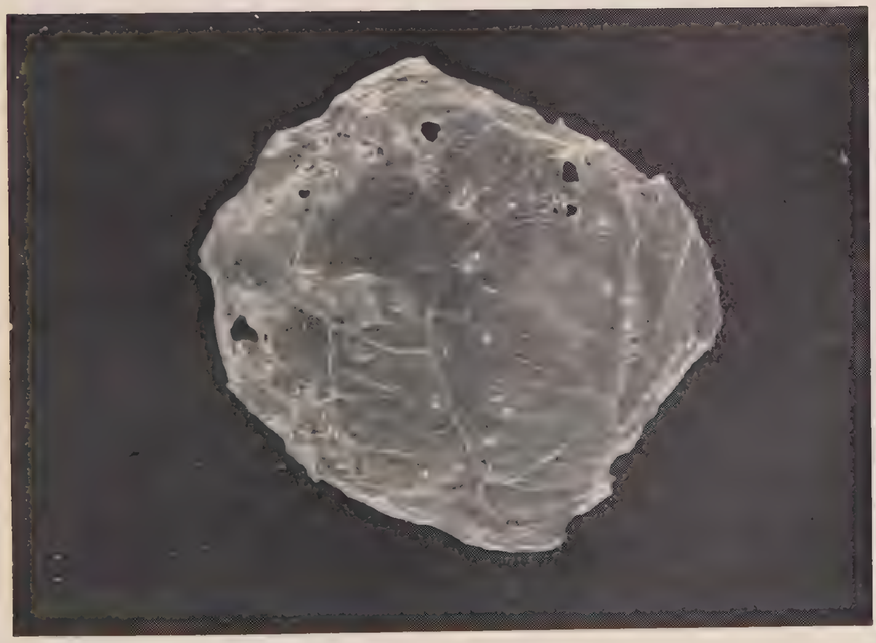

Fig. 2

P. Wildy—Recombination with herpes virus. Plate 1

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Beta-oxidation of Fatty Acids by *Nocardia opaca*

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SUMMARY: A study of the mechanism of breakdown of ω-phenyl-substituted fatty acids by *Nocardia opaca* has been made. Acids with an odd number of carbon atoms in the side chain (phenylpropionic, phenylvaleric and phenylheptylic acids) were converted to benzoic acid, and cinnamic acid was an intermediate. *o*-Hydroxyphenylacetic acid was identified as a common product when acids with an even number of carbon atoms (phenylacetic, phenylbutyric, phenylcaproic and phenylcaprylic) were used. This evidence supports β-oxidation as a mechanism of breakdown of short chain fatty acids by *N. opaca*.

It was shown that *Nocardia opaca* would grow on a wide range of saturated aliphatic fatty acids as sole carbon and energy source (Webley, 1954), and that washed suspensions of glucose grown cells gave enhanced oxygen uptake in the presence of these acids (Webley & DeKock, 1952). The mechanism by which acid-fast organisms attack fatty acids is, at present, unknown. In the present work the metabolism of ω-phenyl-substituted fatty acids by *N. opaca* has been studied and the results are in favour of β-oxidation of the short chain fatty acids by this organism. Growth experiments, manometric measurements, spectrochemistry and chromatography have been employed.

METHODS

Organism. Nocardia opaca, strain T_{16} was used throughout (Webley & DeKock, 1952).

Growth experiments. These were carried out as previously described (Webley, 1954), but with substrates at 0·01 M concentration, unless otherwise stated.

Production of cells for oxygen uptake measurements, etc. Organisms were grown on liquid medium as previously (Webley & DeKock, 1952), but the shaking machine of Webley & Duff (1955) was used. Medium containing 2% (w/v) glucose was used in 200 ml. lots in 1 l. Pyrex culture flasks (Jobling Cat. No. 1410). Incubation was at 25° for 4–6 days.

Manometric experiments. Measurements of O_2 uptake were carried out as before (Webley & DeKock, 1952), substrates being tipped in from the side arm of the Warburg flasks after the overnight incubation required to decrease endogenous activity. For adaptation experiments the 'exposure' technique of Silliker & Rittenberg (1951) was used. For this purpose phenyl-substituted fatty acids (0·4 μmole/ml.) were added to the suspensions of the glucose-grown cells at the beginning of the overnight incubation period. Before substrates were tipped in next morning readings were taken for 30 min. to ensure that the endogenous respiration in each vessel was the same. The rate rarely varied more than 10% from one vessel to the next. Readings were taken at 10 min. intervals after addition of the substrates.

Larger scale experiments for detection of intermediates. These were performed on the shaking machine used for the production of the cells. The same type of flask was employed containing 100 ml. of 0·0133 M-phosphate (pH 7·2), 5–25 μmole/ml. phenyl substituted acid (as sodium salt) and 200–400 mg. (dry weight) of washed glucose-grown cells. Strict aseptic conditions were observed as the experiments were sometimes continued over a number of days. Samples were withdrawn periodically when required, centrifuged and examined spectrochemically as described below. Chromatographic analyses were performed when desirable during the course or at the end of the experiment when the whole contents of the flasks were used.

Spectrochemical techniques. The ultraviolet absorption spectra of samples taken from the large-scale experiments were determined in both acid and alkaline solution. Absorption measurements were made at 5 mμ. intervals between 200 and 350 mμ. using a Beckman model D.U. spectrophotometer. The estimation of benzoic and cinnamic acids in the presence of ω-phenyl substituted fatty acids depends on the intense absorption maxima of benzoic acid at 229 mμ. ($\epsilon = 1{\cdot}10 \times 10^4$) and of cinnamic acid at 278 mμ. ($\epsilon = 2{\cdot}04 \times 10^4$). At these wavelengths the phenyl substituted fatty acids show only weak absorption. The estimation of phenols formed from the substituted fatty acids depends on the rise in absorption which occurs with simple phenols on passing from acid to alkaline solution. For this purpose absorption measurements at 290 and 240 mμ. were made in acid and alkaline solution. *o*-Hydroxyphenylacetic acid was used as a standard.

Paper chromatography. Unidimensional paper chromatography was carried out by the descending technique with stainless steel troughs enclosed in bell jars accommodated in a chamber thermostatically controlled at 23°. Organic acids were identified by the method of Fewster & Hall (1951) with ammonia + ammonium carbonate buffered *n*-butanol as developing solvent and a buffered solution of bromophenol blue and methyl red as a spray. Unsaturated acids were detected as white spots on a light reddish purple background when the chromatogram was sprayed with a dilute aqueous solution (0·1–0·5%, w/v) of potassium permanganate. Phenols and phenolic acids were detected by the diazotized sulphanilic acid spray of Evans, Parr & Evans (1949). The developer of Fewster & Hall (1951) gave good separations with these compounds, and was rather more convenient than the butanol + pyridine + saturated NaCl solution used by Evans *et al.* (1949).

In general, cells from large-scale experiments were separated by centrifuging, and the acidified (HCl) supernatant solution was extracted six times with small amounts of ether in a separating funnel. The ether was removed and the residue dissolved in a few drops of acetone and applied to the origin of the chromatogram. Normally a standard substance was applied to each side of the paper and the unknown material spotted in the centre. A very small difference in R_F value could be detected in this way.

RESULTS

Experiments with benzoic acid and odd numbered phenyl-substituted fatty acids

Nocardia opaca did not grow with benzoic acid as carbon source. Similarly, very little growth was obtained on phenylpropionic (PhC-3) phenylvaleric (PhC-5) and phenylheptylic (PhC-7) acids (Table 4). However, washed cell suspensions of *N. opaca* showed no increased oxygen uptake in the presence of benzoic acid but gave marked increases with the other compounds (Table 1).

Table 1. *The oxygen uptake of* Nocardia opaca *in the presence of benzoic, cinnamic, phenylpropionic, phenylvaleric and phenylheptylic acids*

Each vessel contained 5·7 mg. dry weight of a washed suspension of glucose-grown cells of *N. opaca*, 0·5 ml. 0·066 M-phosphate (pH 7·2) and distilled water to give 2·0 ml. After overnight incubation 0·5 ml. of distilled water or 0·01 M-sodium salts of the acids were tipped in from the side arm.

Addition	O_2 uptake in 180 min. (μl.)
None	121
Benzoate	129
Cinnamate	198
Phenylpropionate	187
Phenylvalerate	237
Phenylheptylate	379

The manometric experiments suggested the possibility that the side chain of PhC-3, PhC-5, PhC-7 was oxidized to benzoic acid as would be expected for β-oxidation. The following experiments showed that this occurred, and that cinnamic acid was produced as an intermediate in the breakdown of these acids to benzoic acid.

The production of cinnamic and benzoic acids from phenylpropionic, phenylvaleric and phenylheptylic acids by washed cells of Nocardia opaca

A culture flask containing 100 ml. 0·0133 M-phosphate (pH 7·2), 25 μmole/ml. phenylpropionate and 372 mg. (dry weight) of glucose-grown cells was set up as described under Methods. Samples were periodically removed, with aseptic precautions, centrifuged and examined spectrochemically. After incubation for 48 hr. the maxima due to benzoic and cinnamic acids are distinct and the sum of the absorptions of the three constituent acids yield the observed 48 hr. spectrum at every wavelength between 210 and 330 mμ. (Fig. 1). Excellent agreement between the observed and the calculated spectrum was also found for alkaline solutions. As the spectra of both benzoic and cinnamic acids change on going from acid to alkaline solution this gives independent confirmation of the presence of these acids. Successive absorptiometric analyses of the phenylpropionic acid solution at intervals over the complete experiment shows that the benzoic acid concentration rises steadily until the substrate is exhausted, and the conversion is approximately quantitative; the concentration of cinnamic acid rises to a maximum and then falls to zero at the end of the experiment (Fig. 2). It is clear, therefore, that cinnamic acid is an intermediate in the oxidation of phenylpropionic acid to benzoic acid by *Nocardia opaca*. Moreover, authentic cinnamic acid (2 μmole/ml.) was oxidized to benzoic acid by *N. opaca*.

The formation of benzoic acid from phenylpropionic acid was finally confirmed by its isolation at the end of the experiment. The cells were removed by centrifuging and the supernatant was acidified and extracted with ether. The ether extract yielded crystalline benzoic acid, which was purified by sublimation and identified by its infra-red spectrum.

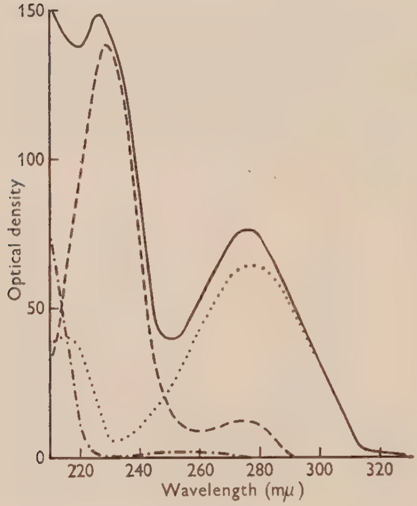

Fig. 1

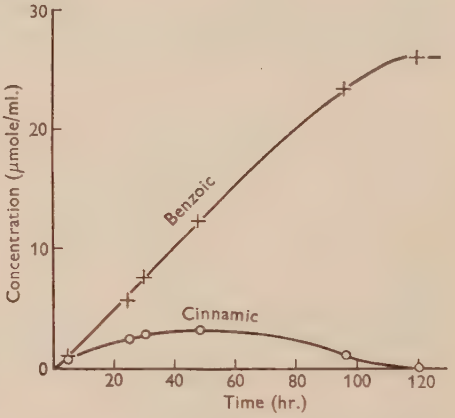

Fig. 2

Fig. 1. Absorption spectrum of a solution, originally containing 25 μmole/ml. phenylpropionic acid, after 48 hr. incubation with *Nocardia opaca* (——). For comparison, the spectra of the separate constituents of the above solution are given as follows: benzoic acid (- - - -), cinnamic acid (. . . . .), and residual phenylpropionic acid (-·-·-·-·).

Fig. 2. Formation of benzoic and cinnamic acids during the oxidation of phenylpropionic acid (25 μmole/ml.) by *Nocardia opaca*.

A similar experiment was terminated after 48 hr. (i.e. at the half-way stage). The contents of the flask were centrifuged and extracted as described under Methods. Paper chromatography of the extract showed the presence of an unsaturated acid substance with an R_F value identical with that of authentic cinnamic acid. Benzoic acid was indicated similarly with the bromophenol blue-methyl red spray.

Phenylvaleric and phenylheptylic acid were also shown by absorptiometric methods to be converted to benzoic acid with formation of cinnamic acid as an intermediate (Table 2). Benzoic acid was formed at a linear rate from phenylpropionic acid (Fig. 2), but there is an increasing lag in its formation as the fatty acid chain lengthens. This observation is in agreement with the supposition that the chain length is reduced stepwise by β-oxidation.

Table 2. *Concentrations of cinnamic and benzoic acids at intervals during the metabolism of phenylvaleric and phenylheptylic acids by* Nocardia opaca

Culture flasks contained 100 ml. 0·0133 M-phosphate (pH 7·2) 10 μmole/ml. phenylvaleric or phenylheptylic acids, and 250 mg. (dry weight) cells of *N. opaca.* 5 ml. samples were withdrawn aseptically at stated intervals.

	Acids present initially			
	Phenylvaleric		Phenylheptylic	
	Acids found (μmole/ml.)			
Time (hr.)	Cinnamic	Benzoic	Cinnamic	Benzoic
0	0	0	0	0
24	1·73	1·09	0·97	0·29
48	4·07	3·53	2·47	1·45
72	2·13	8·15	4·38	3·79
96	0	10·65	1·60	7·55
144	0	10·70	0	10·20

Phenylundecanoic acid was toxic in manometric experiments at concentrations exceeding 0·001-M. It increased the oxygen uptake at this concentration (Table 3). For comparison phenylcaprylic acid and phenylpropionic acid were included. The experiment was continued overnight, and the following morning the contents of the vessels were centrifuged and examined spectrochemically. Phenylpropionic acid had been converted to benzoic acid but no trace of the latter could be found in the experiment with undecanoate. Thus the breakdown of phenylundecanoic acid differs from that of the other odd-numbered phenyl substituted fatty acids used. This is of interest in view of the work of Mahler (1953) with animal systems where the enzyme that activates C_4–C_{10} fatty acids caused a slow activation of the C_{11} and did not activate the C_{12} acid at all.

Table 3. *The oxygen uptake of* Nocardia opaca *in the presence of phenylpropionic, phenylcaprylic and phenylundecanoic acids*

Each vessel contains 20 mg. dry weight of a washed suspension of glucose-grown cells of *N. opaca*, 0·5 ml. 0·06 M-phosphate (pH 7·2) and distilled water to give 2·25 ml. After overnight incubation 0·25 ml. of distilled water or 0·01 M-sodium salts of the acids were tipped in from the side arms.

Addition	O_2 uptake in 180 min. (μl.)
None	63
Phenylpropionate	78·8
Phenylcaprylate	177·6
Phenylundecanoate	127·7

Experiments with phenylacetate and even-numbered phenyl-substituted fatty acids

In contrast to its behaviour with benzoic acid and the odd-numbered phenyl-substituted fatty acids *Nocardia opaca* grew well on phenylacetic acid (PhC-2), phenylbutyric acid (PhC-4) and phenylcaproic acid (PhC-6). There was a time lag before growth commenced on PhC-4 and PhC-6, this being longer with the latter (Table 4). Phenylcaprylic acid (PhC-8) did not support growth at concentrations greater than 0·001-M.

Table 4. *Growth of* Nocardia opaca *on phenyl-substituted fatty acids*

Growth in mineral salts with 0·01 M-sodium salts of the acids as substrates. Incubated at 25° and examined periodically. 0=no growth; (+)=very slight growth; + =definite growth; +++to ++++++=good growth.

Substrate	Growth (days)			
	3	13	23	36
Benzoate	0	0	0	0
Cinnamate	0	0	(+)	(+)
Phenylacetate	+++	++++	+++++	++++++
Phenylpropionate	0	0	(+)	(+)
Phenylbutyrate	+	+++	++++	+++++
Phenylvalerate	0	(+)	(+)	(+)
Phenylcaproate	0	+	+++++	+++++
Phenylheptylate	0	0	0	(+)

In manometric experiments glucose-grown cells gave increased oxygen uptake in the presence of phenylacetate, phenylbutyrate, phenylcaproate (Table 5), and phenylcaprylate (Table 3). Also an adaptive curve was obtained with phenylacetate but not with phenylbutyrate (Fig. 3 (*a*)). The other phenyl substituted acids behaved similarly to phenylbutyrate. Oxidation of phenylacetate by glucose-grown cells occurred without a lag if they were first incubated with PhC-4, PhC-6 or PhC-8 (Fig. 3*b*), but the odd-numbered members PhC-3, PhC-5, PhC-7 were ineffective. This supports the view that the oxidation of phenylbutyrate, phenylcaproate and phenylcaprylate proceeds via phenylacetate as would be expected for β-oxidation.

Attempts to detect phenylacetic acid during the metabolism of the even-numbered phenyl substituted acids were not successful. However, evidence was obtained of the production of a phenolic compound (*o*-hydroxyphenylacetic acid) common to the metabolism of phenylacetic, phenylbutyric, phenylcaproic and phenylcaprylic acids.

The production of o-hydroxyphenylacetic acid from phenylacetate, phenylbutyrate, phenylcaproate and phenylcaprylate by washed cells of Nocardia opaca

Culture flasks containing either 20 μmole/ml. phenylacetate, phenylbutyrate, 10 μmole/ml. phenylcaproate, or 5 μmole/ml. phenylcaprylate and 339 mg. (dry weight) of glucose-grown cells were set up as described under Methods.

The experiment with PhC-2, PhC-4, PhC-6 was concluded after 7 days but that with PhC-8 continued for 12 days. After centrifuging, a sample (2 ml.) was taken for spectrochemical analysis and the remainder acidified and extracted with ether as previously described. Paper chromatography of the extracts showed that phenols were present in all cases and pure specimens were obtained by ether extraction of the spots from six papers in a Soxhlet apparatus. It was necessary to spray the papers with N-hydrochloric acid before extraction. The main phenolic product in all cases was *o*-hydroxyphenylacetic acid. It had the same R_F value and the spots were identical with an authentic sample in three different developing solvents: *n*-butanol + acetic acid + water (4 : 5 : 5), *n*-butanol + pyridine + saturated aqueous sodium chloride solution + ammonia sp.gr. 0·880 (4 : 8 : 5 : 3) (Bray, Lake, Thorpe & White, 1950), and the buffered ammonia + ammonium carbonate solvent of Fewster

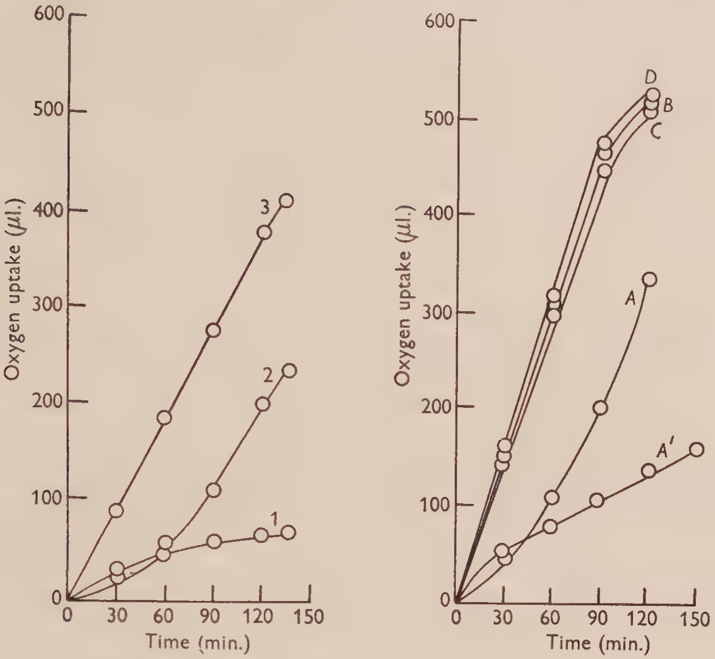

Fig. 3. (*a*) Adaptive patterns of glucose-grown cells of *Nocardia opaca* with the following compounds: 1, no substrate; 2, phenylacetate; 3, phenylbutyrate. Each vessel contained 20 mg. dry weight of cells and 2 µmole/ml. of the respective substrate. (*b*) Effect on phenylacetate oxidation of exposure of glucose-grown cells to distilled water (A and A′), phenylbutyrate (0·4 µmole/ml.) (B), phenylcaproate (0·4 µmole/ml.) (C), and phenylcaprylate (0·4 µmole/ml.) (D). Phenylacetate (2 µmole/ml.) added subsequently to A, B, C and D. Distilled water added to A′.

Table 5. *The oxygen uptake of* Nocardia opaca *in the presence of phenylacetic, phenylbutyric and phenylcaproic acids*

Each vessel contained 5·7 mg. dry weight of glucose-grown cells of *N. opaca*, 0·5 ml. of 0·66 M-phosphate (pH 7·2) and distilled water to give 2·0 ml. After overnight incubation a further 0·5 ml. of distilled water or 0·01 M-solutions of the sodium salts of the acids were tipped in from the side arm.

Addition	O_2 uptake in 180 min. (µl.)
None	119
Phenylacetate	194
Phenylbutyrate	241
Phenylcaproate	330

& Hall (1951). The R_F values were 0·95, 0·74 and 0·48 respectively. *o*-Hydroxyphenylacetic acid was well separated from the para isomer. The main spot given by the extracted material showed both phenolic and acidic properties when sprayed with the appropriate reagents. *o*-Hydroxyphenylacetic acid was the only phenolic product obtained from phenylacetic and phenylcaprylic acids but a second phenolic acid (R_F 0·40 in buffered ammonia + ammonium carbonate solvent) was present in the extracts from the phenylbutyrate and phenylcaproate cultures.

Examination of the ultraviolet absorption spectra of the centrifuged media at the conclusion of the experiment showed that approximately 3 % of phenylacetate, 12 % of phenylbutyrate, 14 % of phenylcaproate and 7 % of phenylcaprylate had been converted to phenolic substances. No trace of benzoic or cinnamic acids could be detected. The production of a common product (*o*-hydroxyphenylacetic acid) from the even-numbered phenyl-substituted fatty acids confirms that their oxidation proceeds via phenylacetate as indicated by the adaptation experiments.

Since the presence of phenylacetate as impurity in the above acids would invalidate this conclusion samples were examined by paper chromatography. One spot only was obtained from each acid and these were very clearly distinguished from phenylacetic acid (by their R_F values). Phenylcapric and phenyllauric acids gave two spots on the paper chromatogram and were not included in the present work. These acids melted over a range of 1° and 2° respectively showing that in this case the paper chromatographic methods were more sensitive to impurities than melting point determinations.

DISCUSSION

It is well established that the enzyme systems involved in the oxidation of the normal fatty acids are the same for the oxidation of the phenyl-substituted fatty acids (Rittenberg & Ivler, 1952; Mahler, 1953).

The production of benzoic acid from odd-numbered phenyl-substituted fatty acids (PhC-3, PhC-5, PhC-7) and *o*-hydroxyphenylacetic acid from even-numbered phenyl substituted fatty acids (PhC-4, PhC-6, PhC-8) supports the view that the mechanism of breakdown of fatty acids by *Nocardia opaca* takes place by β-oxidation (cf. Knoop's classical work (Breusch, 1948)). The detection of cinnamic acid from PhC-3, PhC-5, PhC-7 is in line with this hypothesis. Although benzoic acid is formed almost quantitatively from the odd-numbered phenyl-substituted fatty acids (Table 2), the yields of *o*-hydroxyphenylacetic acid from even-numbered acids are relatively low (< 15 %). This phenol appears to arise from a side reaction in the main metabolic pathway of phenylacetic acid. It is not attacked by *N. opaca* and therefore accumulates in the medium. The remainder of the phenylacetate is rapidly consumed and the metabolic pathway by which this is achieved is under investigation.

The production of *o*-hydroxyphenylacetic acid from phenylacetate by *Nocardia opaca* is of interest in the light of its occurrence during penicillin formation (Pan, 1955).

The work of Silliker & Rittenberg (1952) with *Serratia marcescens* using the technique of simultaneous adaption suggests that *β*-oxidation does not occur in this organism. Our strain of *Nocardia opaca* differs from the strain of *Serratia marcescens* used by these workers in that the oxidation of fatty acids by the latter organism is catalysed by adaptive enzymes. No adaptive mechanism was observed with *Nocardia opaca*. Further, cells of *Serratia marcescens* which can oxidize higher fatty acids do not oxidize propionic, butyric or valeric acids due to 'impermeability of the living cells to these acids' (Silliker & Rittenberg, 1951). In view of these major differences, and because in *Nocardia opaca* we are dealing with an acid-fast organism with a pronounced lipid metabolism it is not surprising that its mode of attack on fatty acids differs from that found in organisms not normally regarded as fat decomposers.

We are indebted to Dr S. C. Rittenberg for his kindness in supplying us with samples of *ω*-phenyl-substituted fatty acids and Dr S. W. Stroud for the gift of some pure *o*-hydroxyphenylacetic acid.

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Studies on the Ability of Light to Counteract the Inactivating Action of Ultraviolet Radiation on Plant Viruses

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SUMMARY: Of seven plant viruses tested, all except tobacco mosaic showed the phenomenon of photoreactivation, i.e. plants exposed to visible light after inoculation with preparations partially inactivated by ultraviolet radiation produced more local lesions than plants kept in darkness. Five strains of tobacco mosaic virus, which differed widely in their pathogenicity and other properties, were tested, but none showed the phenomenon. Of the six viruses that did, potato X showed it much the most strongly, tomato bushy stunt and a tobacco necrosis virus the least; cabbage black ringspot, cucumber mosaic and tobacco ringspot were intermediate.

Photoreactivation does not occur immediately after plants are inoculated. With irradiated preparations of potato virus X, most particles need about 30 min. to reach the state in which their ability to infect is affected by light; once in this state, exposure to daylight for 15 min. gives almost complete photoreactivation. Some irradiated particles respond to light of 80 f.c., but others need brighter light; no additional response occurs when light intensity is increased above 600 f.c. The sensitive state of potato virus X persists for about 1 hr. in plants kept in darkness, after which the particles seem to be permanently inactivated.

With antibodies and enzymes, a constant amount of absorbed energy decreases the activity of a unit weight by a given fraction. This rule does not apply to plant viruses, which lose infectivity with less absorbed energy than the rule predicts. If viruses are more sensitive because they contain nucleic acid, their sensitivity is not a direct function of their content of nucleic acid. Potato virus X has the same nucleic acid content as strains of tobacco mosaic virus, but is inactivated by less absorbed energy and individual strains of tobacco mosaic virus also differ by factors of two in the amount of radiation needed to decrease their infectivity by a given fraction.

Visible light mitigates the damaging effects that ultraviolet radiation has on many kinds of cells (Kelner, 1949, 1951; Bawden & Kleczkowski, 1952). It can also partially restore the activity of irradiated preparations of some viruses; exposure of the irradiated viruses to light *in vitro* has no effect on their ability to multiply, but more of the irradiated particles multiply when recently infected cells are exposed to light than when they are kept in darkness (Dulbecco, 1950; Bawden & Kleczkowski, 1953). The alleviating effect of visible light both on irradiated cells (Kelner, 1949) and bacterial viruses (Dulbecco, 1950) has been called 'photoreactivation', and for simplicity and uniformity we shall also use this term. However, we stress the fact that there is no evidence to show whether the phenomenon occurs because changes caused in virus particles by the radiation are reversed in illuminated cells, or because conditions in such cells favour infection and allow damaged particles to multiply.

Of the three viruses we studied earlier, tomato bushy stunt and the Rothamsted tobacco necrosis virus showed the phenomenon of photoreactivation, and tobacco mosaic virus did not (Bawden & Kleczkowski, 1953). As the same host plant (*Nicotiana glutinosa*) was used for tests with tomato bushy stunt and tobacco mosaic viruses, it seemed that the ability to respond to visible light was an intrinsic property of individual viruses and was not determined by the identity of the host. Tomato bushy stunt and the tobacco necrosis virus resemble one another in many respects; both have spherical particles containing about 18% nucleic acid and have small temperature coefficients of thermal inactivation, whereas tobacco mosaic virus has elongated particles, only a third as much nucleic acid and a large coefficient of thermal inactivation. To see how general photoreactivation is with plant viruses and whether its occurrence can be correlated with any other properties, such as shape or gross composition, we have now studied four other viruses and four additional strains of tobacco mosaic virus. For these experiments we were restricted to viruses that occur in relatively high concentration in expressed sap and produce countable local lesions, but within these limitations we made a choice with as wide a range of characters as possible. Potato virus X has the same nucleic acid content as tobacco mosaic and behaves in the same manner when heated; it differs in being hydrolysed by proteases and its elongated particles are thinner and more flexible. Cabbage black ringspot virus has particles morphologically similar to those of potato virus X; their content of nucleic acid is unknown. The spherical particles of tobacco ringspot virus are about the same size as those of tomato bushy stunt but contain more nucleic acid. Cucumber mosaic virus, which is also probably spherical, has a small temperature coefficient of thermal inactivation; its content of nucleic acid is unknown.

Photoreactivation was not detectable with any of the strains of tobacco mosaic virus, but the other viruses all showed it to a greater extent than did tomato bushy stunt or the tobacco necrosis virus, and potato X, despite its many similarities to tobacco mosaic virus, showed it far more strongly than any other. Thus we were unable to correlate the phenomenon with other known distinguishing features of plant viruses. The viruses which show it are so diverse that photoreactivation is almost certainly usual rather than exceptional, although it would be premature to assume that tobacco mosaic virus is a unique exception. As the phenomenon seems to be general, and potato virus X provided such a convenient test object for studying details of the process, the main purpose of our work was changed to find out more about the conditions in which visible light permits the multiplication of virus particles after they have been altered by ultraviolet radiation.

MATERIALS AND METHODS

The five strains of tobacco mosaic virus used were: (1) the Rothamsted stock culture; (2 and 3) U1 and U2 (Ginoza, Atkinson & Wildman, 1954), which were kindly supplied by Dr S. G. Wildman; (4) one that causes a systemic mosaic in *Datura stramonium* and which we shall call the Datura strain; (5) tomato aucuba mosaic virus. The first four were propagated in tobacco (*Nicotiana tabacum*), U2 in Turkish, as it gives only necrotic local lesions in

White Burley, and the others in White Burley; local-lesion tests were made with all four in *Nicotiana glutinosa*. Tomato aucuba mosaic virus was propagated in tomato (*Lycopersicum esculentum*, var. Kondine Red) and local-lesion tests were made with White Burley tobacco. White Burley tobacco was also used for local-lesion tests with the other four viruses, two of which, tobacco ringspot and cucumber mosaic were propagated in tobacco, the third, potato X, in *Nicotiana glutinosa*, and cabbage black ringspot in turnip (*Brassica napus*).

Most of the experiments were made with clarified infective sap diluted in M/15-phosphate buffer (pH 7). The sap extracted from macerated leaves was clarified by freezing, thawing and centrifuging at 6000 r.p.m. Purified preparations of the strains of tobacco mosaic virus and of potato virus X were made by repeatedly precipitating the viruses at their isoelectric points or with $(NH_4)_2SO_4$, followed by sedimenting them from neutral solutions by centrifuging at 40,000 r.p.m. When preparations of the tobacco mosaic viruses were still coloured, the colour was removed by incubating them with citrate (Ginoza *et al.*, 1954). The purified preparations were dialysed against distilled water and diluted in the buffer before being exposed to ultraviolet radiation.

Ultraviolet radiation was provided by a Vitreosil low-pressure mercury lamp, most of whose radiation is of wavelength 2537 A. The fluids were irradiated as layers 0·14 cm. deep in Petri dishes placed 20 cm. from the lamp, where the intensity of the radiation was 870 μW./sq.cm. The dishes were rocked continually during the period of irradiation; this was assumed to have ensured that all particles were equally exposed.

The amount of radiation at wavelength 2537 A. absorbed by fluids was measured with a quartz spectrophotometer.

The relative infectivities of different virus preparations were measured by counting local lesions. When the maximum accuracy was needed, irradiated and un-irradiated preparations at various dilutions were inoculated to half leaves, the different inocula being distributed in such a way that each occurred the same number of times on each plant and at each leaf position. After counting the lesions, a dilution curve was drawn by plotting the numbers of lesions produced by the un-irradiated virus preparation against the logarithm of its dilution. The residual infectivity of irradiated preparations was then obtained by graphic interpolation. When less accurate comparisons sufficed, irradiated and un-irradiated preparations were inoculated to opposite half leaves, after so adjusting the dilutions of the two that they could be expected to give approximately similar numbers of lesions: for instance, if the irradiation was expected to destroy 99 % of infectivity, the irradiated preparation was inoculated 100 times as concentrated as the control.

Unless otherwise stated, all the plants used in any one experiment were treated uniformly before they were inoculated and, to increase their susceptibility to infection, they were usually kept in darkness for 24 hr. immediately before they were inoculated. Immediately after inoculation they were differentially treated, some being placed in daylight or artificial light and others in the dark for different periods. After 24 hr. all the plants were again returned to the ordinary daylight conditions of a glasshouse.

RESULTS

The photoreactivation of different viruses

The course of inactivation by ultraviolet radiation of all viruses previously studied approximated closely to that of a first-order reaction, and this was so whether or not the inoculated plants were placed in the light (Bawden & Kleczkowski, 1953). We have not made critical tests with the other viruses used in this work, but their inactivation also seems to approximate to a first-order reaction. This being so, it can be assumed that the proportion, p, of the original infectivity that remains after a preparation has been irradiated for time t will be given, at least approximately, by the equation $p=e^{-kt}$, in which k is a constant. Using viruses that show the phenomenon of photoreactivation, k will be greater when inoculated plants are placed in the dark than when they are placed in the light, and the ratio of the values obtained with plants in the two conditions will provide a measure of the extent of photoreactivation.

Table 1. *Relative amounts of photoreactivation with different viruses*

Virus	k^*_{dark}	k_{light}	$\frac{k_{\text{dark}}}{k_{\text{light}}}$	Kind of preparation irradiated
(1) Tobacco mosaic				
(*a*) Stock strain	1·6	1·6	1·0	0·02 % solution of purified virus
(*b*) Datura strain	1·6	1·6	1·0	
(*c*) U1	1·6	1·6	1·0	
(*d*) U2	3·3	3·3	1·0	
(*e*) Tomato aucuba mosaic strain	0·7	0·7	1·0	Infective sap at 1/50
(2) Tomato bushy stunt	—	—	1·2†	—
(3) Rothamsted tobacco necrosis	—	—	1·2†	—
(4) Cucumber mosaic	3·2	2·1	1·5	Infective sap at 1/10
(5) Tobacco ringspot	3·2	1·7	1·9	Infective sap at 1/10
(6) Cabbage black ringspot	3·0	1·5	2·0	Infective sap at 1/5
(7) Potato X	2·5	0·8	3·1	Infective sap at 1/10

* Value of k was derived from $p=e^{-kt}$ where p is the proportion of original infectivity remaining after irradiating for t min.: k_{dark} is value when plants were placed in the dark after inoculation and k_{light} when placed in the light. A figure of more than 1 for $k_{\text{dark}}/k_{\text{light}}$ indicates photoreactivation.

† Results obtained with purified viruses and already published (Bawden & Kleczkowski, 1953).

Table 1 gives values for k, and for the ratios of $k_{\text{dark}}/k_{\text{light}}$, obtained from experiments with five strains of tobacco mosaic virus and with six other viruses. The strains of tobacco mosaic virus differ in their sensitivity to ultraviolet radiation, but all give a value of 1 for the ratio $k_{\text{dark}}/k_{\text{light}}$, showing no detectable photoreactivation, whereas with all the other viruses the value is more than 1 and reaches 3 with potato virus X. The ratios in Table 1 are calculated from the results of single experiments and are to be regarded as convenient indications of the extent to which the different viruses can be photoreactivated rather than as exact measures, for the amount of photoreactivation given by one of the viruses in the same host varies from experiment to experiment. With potato virus X, for example, the ratio is usually about 3, but it has occasionally been as small as 2. The extent of photoreactivation does not depend on the identity of the host plant, for the tomato aucuba strain of tobacco mosaic virus showed none in either *Nicotiana glutinosa* or tobacco, whereas potato virus X showed it as strongly in *N. glutinosa* as in tobacco.

We do not know what causes the quantitative variations in response in different experiments, but we suspect that the physiological condition of the plants at the time they are inoculated is important. When plants are placed in the dark for a day or more before they are inoculated, their susceptibility to infection increases, so that any inoculum produces more local lesions than when the plants have been kept under normal glasshouse conditions. Putting plants in the dark after they have been inoculated with tobacco mosaic, tomato bushy stunt or tobacco necrosis viruses, has little effect on the numbers of lesions produced by unirradiated inocula, usually decreasing them slightly (Bawden & Roberts, 1948). Our experiments have confirmed these results with these three viruses, but have given different results with the other four. Putting tobacco plants in the dark for a day or so before they were inoculated always increased the numbers of lesions produced by a given inoculum, though the increase was sometimes small, but putting them in the dark after inoculation gave very variable results. Sometimes plants inoculated with un-irradiated preparations of potato X, cucumber mosaic or tobacco ringspot virus, gave similar numbers of lesions in the light and dark, sometimes plants placed in the dark gave many more lesions than those in the light, and occasionally they gave many fewer. In all experiments to compare irradiated and un-irradiated preparations of these viruses, there was unequivocal evidence of photoreactivation with the irradiated inocula, but the ratio $k_{\text{dark}}/k_{\text{light}}$ varied with the effects of darkness on the numbers of lesions produced by the un-irradiated inocula.

At first sight the failure of strains of tobacco mosaic virus to show any photoreactivation suggests that ultraviolet radiation changes it differently from the others, but this is not a necessary conclusion. With those viruses that show the phenomenon, irradiation obviously produces at least two changes: one that allows particles to multiply only if the cells they infect are illuminated, and another that prevents them from multiplying even in these cells. There is no need to assume that the first change is an essential preliminary to the second; the two may be quite independent happenings. To detect the two, however, the one leading to photoreactivation must occur quicker than the change leading to full inactivation, and the greater the difference between the rates of the two changes, the more easy will the two be to detect. With tobacco mosaic virus, the two kinds of change may also occur, but the one that shows as photoreactivation with the other viruses may be undetectable because it happens only after virus particles have been inactivated. If this is so, however, the kind of change that leads to photoreactivation with other viruses occurs in tobacco mosaic virus only after very much more irradiation, for most of its strains resist inactivation by ultraviolet radiation more than do the other viruses. Ginoza *et al.* (1954) found that the strain U2 is more readily inactivated than other strains tested and we have confirmed this: it shows in

Table 1 by the greater value of k obtained with purified preparations of this strain than with preparations of other strains, when irradiated at the same concentration. U2 has the same extinction coefficient, about 2·75, as the other strains.

The values of k given in Table 1 for viruses irradiated in infective sap cannot be taken as indicating their relative susceptibility to inactivation, because their relative concentrations were unknown and other constituents of the sap also absorb ultraviolet radiation strongly; these other constituents occur in widely different amounts in different samples of sap from the same plant species. For comparing results with different viruses irradiated in infective sap, only the ratios $k_{\text{dark}}/k_{\text{light}}$ are significant as indicating the relative amounts of photoreactivation they give.

Effect of different exposures to light on photoreactivation of potato virus X

The experiments described so far were all made to find whether or not a given inoculum could be photoreactivated. In each one, the plants were treated uniformly before they were inoculated and immediately afterwards were placed either in the light or dark where they were kept continuously for at least 6 hr. The results provide no information on the details of the processes involved in photoreactivation, either about how it is affected by light intensity and the physiological state of plants when they are inoculated, or at what stage during the process of infection it occurs.

To study such details we chose potato virus X because it gives much the largest ratio of $k_{\text{dark}}/k_{\text{light}}$. The effects of irradiation in different experiments were kept as constant as possible by always using the same batch of sap from systemically infected *Nicotiana glutinosa*. The sap was divided into 5 ml. lots stored at −30°, and a freshly thawed lot was used for each experiment. After centrifuging, the sap was diluted 1/10 with M/15-phosphate buffer (pH 7), when it was centrifuged again to remove the precipitate that formed. The clear fluid was then irradiated for 3 min., diluted with an equal volume of the buffer and used as inoculum. This exposure to ultraviolet radiation decreased the infectivity to about 0·05 % that of the original fluid when inoculated plants were placed in the dark and to about 5 % when they were placed in the light for the 6 hr. after inoculation. So that the un-irradiated control should give reasonably comparable numbers of lesions, the sap diluted 1/10 was diluted a further 1/200 with the buffer before using it as an inoculum. The irradiated inoculum then usually produced more lesions than the un-irradiated on plants kept in the light and fewer on those kept in the dark. The two inocula were rubbed over the opposite halves of the same leaves, three leaves on each of three plants being used for each comparison. The ratios of the numbers of local lesions conveniently indicate the relative infectivities of the two inocula, but they are not exact measures of their relative contents of infective virus because numbers of local lesions are not directly proportional to the concentration of infective particles.

To see whether photoreactivation is affected by the metabolism of the plants immediately before they are inoculated, batches of plants were divided into two groups, one of which was kept in the dark for the 48 hr. before the plants they were inoculated and the other continuously in light, namely daylight supplemented by 500 f.c. of artificial light during the two nights. After rubbing opposite half leaves with irradiated and un-irradiated inocula, half of the plants from each group were placed in daylight and half in the dark. Table 2 shows that, regardless of their previous treatment, approximately the same amount of photoreactivation was given by plants placed in the light after they were inoculated. Thus the phenomenon seems to depend on some almost immediate response to light by a host system and not on some stable intermediate product that is synthesized in the light and can persist in plants kept in the dark. The system operates equally readily whether it has previously been continuously active or inactive for long periods, and it is distinct from the system which increases the susceptibility of plants to infection with un-irradiated viruses when they are kept in the dark before inoculation.

Table 2. *Photoreactivation unaffected by treatment of plants before inoculation*

Inoculum	Treatment before inoculation	Treatment after inoculation: Light	Treatment after inoculation: Dark
Control virus X	Light	265*	186
Irradiated virus		1000	32
Control virus X	Dark	305	228
Irradiated virus		1220	54

* Figures are the total number of lesions produced on nine half-leaves. Control inoculum was infective sap at 1/2000; sap at 1/10 was exposed to ultraviolet radiation for 3 min., and then diluted ½ before inoculating.

Table 3. *Photoreactivation of potato virus X after keeping inoculated plants in darkness for different periods*

The time in darkness after inoculation (hr.)	Inoculum	Total nos. of lesions on nine half-leaves	Ratio: irradiated/control
0	Control	269	1·7
	Irradiated	460	
1	Control	292	1·4
	Irradiated	406	
2	Control	425	0·5
	Irradiated	210	
3	Control	420	0·2
	Irradiated	88	
24	Control	317	0·1
	Irradiated	30	

Irradiated virus particles do not remain continuously in inoculated leaves in the state where their ability to infect is affected by visible light. Table 3 shows that 1 hr. in the dark after inoculation had little effect on the amount of photoreactivation that can be obtained, but a stay of 2 hr. greatly decreased it and after 3 hr. there was very little.

Table 4 gives the results of an experiment to find how varying the length of exposure to daylight affects the amount of photoreactivation when the exposures were made at two different times, one immediately after the plants were inoculated and the other after spending a preliminary period of an hour in the dark. The fact that exposures of less than 1 hr. gave more photoreactivation after 1 hr. in the dark than immediately, suggests that the irradiated particles do not immediately become susceptible to the beneficial effects of light. Using the ratios of the numbers of lesions produced by the two inocula to indicate the amount of photoreactivation, it seems that 1 hr. after inoculation most of the particles in plants kept dark are in a condition in which they are susceptible to the effect of light, and that some of these then respond to exposures as short as 7·5 min. and the majority to an exposure of 15 min., exposures which have little or no effect when given immediately after inoculation. To get the same response as is given by 15 min. of exposure after 1 hr. in the dark, 45 min. of exposure are needed immediately after inoculation. This means that about 30 min. pass after inoculation before most of the virus particles reach a state in which they become susceptible to the effects of light, a conclusion further supported by the fact that 30 min. of exposure after 1 hr. in the dark gave approximately the same amount of photoreactivation as 1 hr. in the light immediately after inoculation. As 1 hr. in the light after 1 hr. in the dark gave almost as much photoreactivation as 6 hr. continuously in the

Table 4. *Photoreactivation with exposures to daylight for various periods immediately after inoculation and after keeping for* 1 *hr. in darkness*

Time of exposure to light immediately after inoculation	Inoculum	Total nos. of lesions on nine half-leaves	Ratio: irradiated/control
0	Control	1520	0·35
	Irradiated	547	
7½ min.	Control	1600	0·25
	Irradiated	381	
15 min.	Control	1640	0·65
	Irradiated	1045	
30 min.	Control	1070	0·90
	Irradiated	955	
1 hr.	Control	1405	1·65
	Irradiated	2340	
6 hr.	Control	760	2·6
	Irradiated	1960	
After 1 hr. in darkness			
7½ min.	Control	1065	0·6
	Irradiated	634	
15 min.	Control	1505	1·2
	Irradiated	1805	
30 min.	Control	1207	1·7
	Irradiated	2045	
1 hr.	Control	1100	2·4
	Irradiated	2640	

light, nearly all the particles must have reached the photoreactivable stage within 2 hr. This happens, too, when the plants are in the light for 2 hr.; periods of continuous light between 1 and 6 hr. were not tested in the experiment recorded in Table 4, but they were in other experiments in which 2 hr. gave more photoreactivation than 1 hr., but 3 and 6 hr. gave only a slight additional response.

Thus the initial stages in the process of infection seem to proceed at much the same rate whether plants are kept in the light or dark, and, by combining the results of experiments such as those shown in Tables 3 and 4, three stages in the condition of infecting particles can be detected and their duration approximately timed. First, there is the stable stage of the virus particles in the inoculum, which persists for some time in inoculated leaves; a few particles move out of this stable stage within 10 min. of being inoculated to leaves, but most need about 30 min. and a few as long as 2–3 hr. The change to the second stage is shown by irradiated particles becoming susceptible to photoreactivation and it happens to most particles between 30 and 90 min. after leaves are rubbed; if the inoculated plants are in the light, it probably lasts for 15 min. or less, but if they are in the dark it can last for 1–2 hr. If, during this time, the particles fail to initiate the processes that lead to virus multiplication, they change to the third stage and lose their activity.

This sequence can profitably be compared with the results Dulbecco (1950) obtained with coli phages. He found that irradiated phage particles become susceptible to photoreactivation immediately they infect bacteria, and that the duration of this susceptible state depends on the metabolic activity of the bacterial culture. When the host cells are ‘active’ (conditions that favour phage multiplication) the susceptible state is brief, but it is prolonged when the bacteria are ‘resting’. Plant viruses normally multiply in cells that have long ceased to divide; all our experiments were made with cells that, for this comparison, resemble metabolically ‘active’ rather than ‘resting’ bacteria. The sequence of events, then, from a state insusceptible to photoreactivation, through a transient susceptible one, is similar, the main difference being that potato virus X takes longer to complete each stage, which is at least partly attributable to the fact that our experiments were made at 20° and those with the phage at 37°.

There is now much evidence that infection by coli phages entails the nucleic acid moiety separating from the protein of the phage particle and that the host bacteria are penetrated by the nucleic acid only (Hershey & Chase, 1952). Hence, only the nucleic acid seems to be concerned in photoreactivation and the sensitive state is reached immediately the nucleic acid is in the host cell. By analogy with phage it is reasonable to assume that the period after inoculation when plant viruses cannot be photoreactivated is the time they take to enter the host cells or, perhaps, some specific component of the cells. It may, too, be the time when the nucleic acid becomes separated from the protein part of the virus particles, but there is no direct evidence that such a separation is a part of the process that leads to successful infection and multiplication by plant viruses. Some support for the idea is perhaps provided by experiments with ribonuclease, and Casterman & Jeener (1955) suggest that this enzyme may prevent tobacco mosaic virus from multiplying in recently inoculated cells by destroying its nucleic acid. Ribonuclease has no permanent effect on plant viruses when incubated with them *in vitro*, but its presence in an inoculum prevents the viruses from becoming established in rubbed leaves (Loring, 1942; Kleczkowski, 1946; Bawden & Kleczkowski, 1948). It can also interfere with the course of infection when applied to leaves shortly after they have been inoculated with viruses. We have rubbed solutions of pancreatic ribonuclease over tobacco leaves at different intervals after they were inoculated with potato virus X and found that this decreases the numbers of lesions up to an hour or more but has little effect after 3 hr. It may be significant that this period roughly coincides with that during which the irradiated virus can be photoreactivated, but it would be decidedly premature to draw any firm conclusions about either the behaviour of the infecting particles or the mechanism whereby ribonuclease prevents them from multiplying. Before this can be done, many more detailed experiments will be needed comparing the behaviour of ribonuclease with other inhibitors of infection that have no ability to hydrolyse ribonucleic acid, and it will be necessary to make the comparisons with plants kept in the light and dark for various periods after they have been inoculated with irradiated and normal virus preparations. That ribonuclease may act by affecting the host plants, rather than by destroying the virus or a part of it, is suggested by the fact that the extent to which it decreases numbers of lesions depends on the species of host plant inoculated; but this again is inconclusive, for conditions in cells of some species may be more conducive than in others for enzyme activity. Work with a Rhizobium phage also provides evidence for an effect on the host cell (Kleczkowski & Kleczkowski, 1954). The multiplication of this phage, which probably contains no ribonucleic acid, is not only inhibited when phage, bacteria and ribonuclease are present simultaneously, but when the bacterial cells are first incubated with the enzyme alone and then freed from it before the phage is added.

The fate of the phage nucleic acid that is not photoreactivated in 'active' bacteria is unknown, but such bacteria contain nucleases, which can reasonably be assumed to digest it. Similarly, plant cells contain nucleases and proteases, and, although these do not hydrolyse most plant viruses *in vitro*, they may be able to hydrolyse them *in vivo* after they have changed to the state in which irradiated particles become susceptible to photoreactivation.

Light intensity and duration of exposure

We have not studied the interaction between intensity of light and the length of exposure necessary to give photoreactivation in any detail, but have a few relevant observations. Most of our experiments were done in daylight, usually in cloudy weather when the light intensity in the glasshouses was *c*. 600 f.c. Exposure to this for 15 min. seems enough to activate all particles which have reached the state in cells when they can respond to light, and exposures when the sun shone and the light intensity in the glasshouses was *c*. 2000 f.c. gave no more photoreactivation than did 600 f.c. In only one experiment have we compared artificial light of different intensities, when inoculated plants which had spent an hour in the dark were then given various lengths of time at either 600 or 80 f.c. before again being put in the dark. Twenty minutes in the weaker light gave some photoreactivation and the amount was not increased by prolonging the exposure to 2 hr.; it was less than that given by 10 min. exposure to the brighter light. The results suggest that irradiated particles may differ in the intensity of light needed to reactivate them, and that prolonged exposure to weak light does not have any cumulative effect comparable to brief exposures to bright light, but more critical experiments would be needed to establish the point.

Relative stability of radiated and un-irradiated virus

Exposure of preparations of a Rhizobium bacteriophage (Kleczkowski & Kleczkowski, 1953) or of chymotrypsin (Kleczkowski, 1954*a*) to ultraviolet radiation decreases their stability, and irradiated preparations lose their residual activity more rapidly than do comparably treated un-irradiated ones. We could not detect such a phenomenon with tobacco mosaic, tobacco necrosis or tomato bushy stunt viruses (Bawden & Kleczkowski, 1953), but these are exceptionally stable viruses and neither the control nor irradiated preparations lost any activity over the period in which they were stored. Potato virus X is less stable and, when stored at 2° in M/15-phosphate buffer (pH 7), loses about 90 % of its infectivity in a week. We could, however, find no evidence that irradiation increases the instability. Table 5 shows that irradiated and control preparations lost approximately the same proportion of their infectivity when stored in these conditions, and photoreactivation was as pronounced 8 days after irradiation as immediately afterwards.

Table 5. *The relative stability of irradiated and un-irradiated preparations of potato virus X*

	Lesions produced on plants kept in			
Inoculum	Darkness after inoculation		Light after inoculation	
Control, fresh sap	1200	$R=0{\cdot}35$	590	$R=2{\cdot}4$
Irradiated fresh sap	400		1400	
Control, stored sap	129	$R=0{\cdot}4$	48	$R=3{\cdot}0$
Stored irradiated sap	55		145	

Virus irradiated as infective sap diluted 1/10 with phosphate buffer, pH 7, and stored for 8 days at 2° in this condition. Control was also stored as sap diluted 1/10. Before inoculation the control solutions were diluted a further 1/200 with buffer and the irradiated solution ½. R = ratio of numbers of lesions produced by irradiated virus to those produced by the control.

Inactivation as a function of the amount of energy absorbed

McLaren, Gentile, Kirk & Levin (1953) found that the quantum yields for the inactivation of various enzymes by ultraviolet radiation, when multiplied by their molecular weights, do not differ much from a mean value of 447;

Kleczkowski (1954*b*) found that this was also true with antibodies. Put another way, this means that, if inactivation follows a first-order reaction, the same amount of energy must be absorbed per unit weight of any of these proteins to decrease their specific activities by a given fraction. For example, to halve the original activity of a milligram of any one requires about 0·75 joule of energy at $\lambda = 2537$ A.

It seems that this rule does not apply to plant viruses. McLaren *et al.* (1953) found that the quantum yield of tobacco mosaic virus was too high, and it is much more resistant to inactivation by ultraviolet radiation than potato virus X, even when inoculated plants are kept in the light, and putting inoculated plants in the dark increases its quantum yield by a further factor of about three. The quantum yield of potato virus X cannot be estimated at all accurately, for the estimation requires knowledge of the particle size, and its filamentous particles vary enormously in length. Fortunately, to find how far the virus deviates from the rule which applies to enzymes and antibodies, the quantum yield need not be estimated, but only the amount of energy needed to halve the infectivity of a milligram of purified virus. Even this measurement will not be accurate, for measurements with the spectrophotometer do not distinguish between energy absorbed and scattered, and virus particles are large enough to scatter much of the incident ultraviolet radiation. Thus the viruses will absorb considerably less than the amount measured and their sensitivity to inactivation will seem less than it really is.

Our measurements were made on purified viruses diluted in M/15-phosphate buffer (pH 7), conditions in which four strains of tobacco mosaic virus and potato virus X all give the same extinction coefficient. Preparations of potato virus X in water give higher extinction coefficients, sometimes twice as great, because the particles aggregate in distilled water and consequently scatter more energy. The total energy, absorbed and scattered by 0·02% (w/v) solutions of virus, needed per milligram to halve the infectivity is 0·02 joule for potato virus X when inoculated plants are placed in the dark and about 0·06 joule when they are placed in the light; for the U2 strain of tobacco mosaic virus it is 0·065 joule, and it is 0·15 joule for U1, the Rothamsted stock strain and the Datura strain. The actual amount of energy absorbed is perhaps only half that measured, but even without taking this into consideration it is quite clear that they all fall far short of the 0·75 joule needed to halve the activity of the other proteins. Even the resistant strains of tobacco mosaic virus need about one-fifth the amount predicted by the rule for other proteins, U2 about one-twelfth and potato virus X from 1/12th to 1/40th, depending on whether the inoculated plants are placed in the light or dark. It is reasonable to assume that this greater sensitivity to ultraviolet radiation reflects the fact that the viruses contain nucleic acid and that changes in the nucleic acid directly affect the processes leading to virus multiplication. If this is so, then it seems that the nucleic acids of different viruses, and of different strains of tobacco mosaic virus, must differ in their sensitivity to inactivation by ultraviolet radiation, for the five purified preparations we used all had the same total amount of nucleic acid.

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The Action of Fibrinogen on certain Pathogenic Cocci

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SUMMARY: The properties of the material responsible for the clumping reaction given by suspensions of coagulase-positive staphylococci when shaken in the presence of fibrinogen were examined and a possible mechanism is suggested for the reaction. Of other bacteria examined only streptococci of groups A, C and G absorbed fibrinogen, and of these relatively few gave clumping, though the cell material responsible for absorption was similar to that found in staphylococci. In staphylococci it is believed that a single substance is responsible for both absorption and clumping, and its properties suggest a peptide complex. The possible usefulness of coagulase-positive cocci in the detection of minute amounts of fibrinogen is indicated.

In an earlier publication (Duthie, 1954) it was shown that the slide test for staphylococcal coagulase depended on substance on the staphylococcal cell wall which combined with the fibrinogen of certain animals, causing clumping of the bacteria when shaken. The reaction was in no way related to that produced in plasma by free coagulase, and the substance on the cell wall was named bound coagulase, although the alternative name 'clumping factor' was suggested. For reasons to be given later the latter name is now thought to be preferable. Clumping of the cells was found to be inhibited by staphylococcal antiserum prepared in either horses or rabbits, and this action of the antiserum was neutralized by material liberated from the cell wall of staphylococci early in autolysis. This crude material was antigenic and had properties, as judged by the antibody inhibition test, which were similar to those of clumping factor. It had no detectable action on fibrinogen. Further studies on clumping factor were clearly desirable and are reported in the present paper.

METHODS

Strains used. Unless otherwise stated, the experiments were carried out with the Newman strain of *Staphylococcus aureus* used previously. Cultures were grown in digest broth, agitated on a gramophone motor, washed and resuspended in saline to a standard density (4×10^9 cells/ml.). Since old cultures or suspensions sometimes gave indistinct clumping at low concentrations of fibrinogen, fresh suspensions were used when possible. Clumping at these low concentrations was often improved by boiling the cells for a few minutes or by adding a trace of bovine albumin.

Other pathogenic bacteria tested were grown in digest broth supplemented when necessary by human serum and glucose. Streptococci and pneumococci were grown in digest broth containing 2% (w/v) peptone, 1% (w/v) glucose and 3% (w/v) sodium glycerophosphate. The pneumococcal cultures were supplemented by 0·2% (w/v) bovine albumin. Cells were spun down and resuspended overnight in thiomersalate 0·05% (w/v) after which they were washed, resuspended in saline and used. Streptococcal suspensions could be stored in saline containing chloramphenicol 0·01% (w/v) for several weeks at 4°.

Plasma or fibrinogen titre. Standard cell suspension (0·2 ml.) was added to 0·2 ml. vol. of increasing dilutions of fibrinogen or plasma in tubes 5·0 × 0·8 cm. The tubes were shaken vigorously for at least 4 min. in a rack mounted horizontally on a 'Microid flask' shaker and were then examined with a hand lens in direct lighting and with a dark background. The degree of clumping was recorded as before (Duthie, 1954). Normal suspensions of the standard strain gave minimal clumping between 1 and $2 \cdot 0 \times 10^{-7}$ (w/v) human fibrinogen or at 1/20,000 (v/v) fresh human plasma final concentrations. Suspensions giving less than minimal clumping or showing trace clumping when shaken in saline were discarded.

Titration of inhibitor or of antibody to bound coagulase. These titrations were carried out as in the previous paper (Duthie, 1954) except that the antiserum used was replaced by horse serum EX 1363 provided by the Wellcome Research Laboratories and which was assigned the arbitrary value of 300 units/ml. Shaking was carried out until the tube receiving the 1/600 dilution of this antiserum, i.e. 0·5 unit/ml., gave faint clumping.

Absorption of antiserum. Volumes (0·1 ml.) of a five-times concentrated standard cell suspension (2×10^{10} cells/ml.) were added to 0·4 ml. volumes of increasing dilutions of antiserum ranging from 5 to 1 unit/ml. and left in contact for 20 min. on the bench, with occasional mixing. The cells were then removed by centrifuging and the supernatants compared with control antiserum to which the same volume of saline had been added. This was done by taking 0·1 ml. volumes and adding 0·1 ml. of the standard suspension. After 20 min. at room temperature 0·1 ml. of bovine fibrinogen (0·2%, w/v) was added and the tubes shaken until the 5 unit/ml. tubes absorbed with the suspension of Newman staphylococci gave minimal clumping. Comparison with tubes absorbed with other suspensions then showed greater or less clumping.

Absorption of fibrinogen. Volumes (0·1 ml.) of a five-times concentrated suspension of staphylococci were added to 0·4 ml. volumes of increasing dilutions of human fibrinogen, ranging usually between 0·002% and 0·0002% (w/v) human fibrinogen. After a few minutes contact on the bench the cells were separated, 0·2 ml. volumes of the supernatants placed in fresh tubes and 0·2 ml. volumes of standard suspension added. The tubes were usually shaken until minimal clumping appeared in the tube containing the least fibrinogen.

Absorption of fibrinogen on cells. Since cells coated with fibrinogen may not clump a further test was provided by washing cells, which had been in contact with fibrinogen, at least twice in saline and resuspending to the standard concentration. When 0·2 ml. of the standard suspension of coagulase-positive staphylococci was added to 0·2 ml. of these washed cells, clumping occurred when fibrinogen was present on their surfaces.

Enzyme preparations. The crystalline trypsin was that used by Duthie & Lorenz (1949). Crystalline pepsin was a 1/60,000 National Formulary, U.S.A. preparation supplied by Armour Laboratories. Papain of unknown potency was supplied by the Nutritional Biochemical Corporation and was activated by the addition of NaCN. Lysozyme supplied by Armour Laboratories had a potency of 10^6 unit/g.

RESULTS

A comparison of the properties of a number of staphylococcal strains from various sources showed a good correlation between ability to absorb both antiserum and fibrinogen and the fibrinogen titre of the organisms (Table 1). Freshly isolated strains from pathological sources almost always possessed these qualities in high degree, old laboratory strains to a more variable and lesser extent, while albus strains from non-pathological sources did not possess them at all. Strains which failed to produce free coagulase as shown by the tube test were unable to absorb antiserum or fibrinogen and did not clump, though the converse was not necessarily true. Decrease in ability to absorb antiserum or fibrinogen or to clump at low concentrations of fibrinogen was not always accompanied by loss of toxic properties, since strain Wood 46 was the well-known producer of haemolysin, strain VL was a good producer of leucocidin and several strains such as 010 and Pusey produced large amounts of free coagulase in the presence of albumin. It should be noted that the Wood 46 strain, used to produce toxoid from which the antiserum used was derived, contained no surface antigen against this antibody, since it failed to absorb it.

Table 1. *Comparison of absorption of antiserum, fibrinogen and the fibrinogen titre of different strains of staphylococci*

	Concentration before absorption											
	Antiserum (unit/ml.)						Fibrinogen (%, w/v)					
	5	4	2	1	0		0·001	0·0005	0·0002			
	After absorption											
Organism	Inhibition of clumping by residual antiserum					Antibody absorbed	Clumping by residual fibrinogen			Fibrinogen absorbed	Fibrinogen titre	Free coagulase production
S. albus	–	–	tr.	+	++	Nil	++	++	++	Nil	Nil	–
Wood 46	–	–	tr.	+	++	Nil	++	++	++	Nil	Nil	±
010	–	–	tr.	+	++	Nil	++	++	++	Nil	Nil	++
VL	–	tr.	+	++	++	Some	++	±	–	Some	10^{-5}	±
Newman	+	+±	++	++	++	Some	+	±	–	Some	10^{-6}	++
Saline control	–	–	–	+	++	Nil	++	++	++	Nil		

++ = gross clumps; +± = clumps; + = moderate clumps; ± = faint clumps; tr. = trace; – = zero result.

Stability of the clumping factor. Since it was uncertain whether or not the absorbing antigen on the cells was identical with clumping factor, parallel measurements were made of the three properties after heating at different pH values, treatment with certain enzyme preparations and finally extraction with fat solvents (Table 2). After treatment the cells were rewashed and suspended to the original volumes. It will be seen from Table 2, which gives examples of these experiments with one strain of staphylococcus, that retention or loss of the three properties measured was well correlated. The clumping material was rapidly removed by pepsin and papain, somewhat more slowly by trypsin at pH 9, old cultures being more resistant than those which were still multiplying before washing. No non-proteolytic enzymes were found which removed either the antigenic or fibrinogen-fixing material. There was no loss in 18 hr. at pH 11 and 20° but a marked loss at 37° in the same time.

In general there was always some loss of the absorbing properties when the cells were heated for more than a few minutes at temperatures above 60°, and this loss increased with rise in temperature and exposure time. This was not always reflected in the clumping titre, since the cells became more unstable and tended to clump slightly in saline and to a higher titre in fibrinogen than did the original suspension. Storage of cell suspensions in saline usually resulted in a loss of all three properties with a progressive loss in stability of the cells in saline. All three properties were, however, stable in cells stored after heating in carbol-saline, although this had caused some initial loss. The cells displayed a remarkable resistance to extraction by fat solvents and there was little loss in their power to absorb either fibrinogen or antiserum following refluxing for 3 hr. in acetone. The resuspended cells were then slightly more unstable than before.

Table 2. *Changes in the properties of suspensions of the Newman strain following various treatments*

	Concentration before absorption										
	Antiserum (units/ml.)						Fibrinogen (%, w/v)				
	5	4	2	1	0		0·002	0·001	0·0005		
	After absorption										Minim concent tion c fibrinog clumpi
Treatment of suspensions	Effect on cell fibrinogen suspension normally giving ++ clumping					Antigen loss	Clumping by residual fibrinogen			Fibrinogen fixing loss	
1. Papain 30 min. pH 7	–	–	–	±	++	Complete	++	++	+	Complete	Nil
2. Pepsin 5 min. pH 2	–	–	–	–	++	Complete	++	++	+	Complete	Nil
3. Trypsin 10^{-3} 2 hr. pH 9·5	–	–	–	±	++	Complete	++	++	+	Complete	Nil
4. Held 2 hr. 37° pH 2	–	–	–	++	++	Complete	++	–	–	Partial	10–7
5. Held 2 hr. 37° pH 9·5	tr.	+	++	++	++	Nil	+	–	–	Nil	10–7
6. Held 5 min. 60° pH 9·5	±	+	++	++	++	Nil	+	tr.	–	Nil	Contro clumpe
7. Held 5 min. 100° pH 9·5	–	–	+	++	++	Partial	+	±	–	Nil	10–7
8. Autoclaving	–	–	tr.	++	++	Complete	++	++	++	Complete	Nil
9. Acetone-ether extraction	–	tr.	++	++	++	Partial	+	–	–	Nil	10–7
10. Heated in 0·5 % carbol saline 60° 30 min.	–	tr.	+±	++	++	Partial	+	tr.	–	Partial	10–7
11. Control cells	±	+	++	++	++	Nil	+	–	–	Nil	10–7
12. Saline control	–	–	–	++	++		++	++	±		

++ = gross clump; +± = clumps; + = moderate clumps; ± = faint clumps; tr. = trace; – = zero resul

Antisera. These were readily produced by the intravenous or intracardiac injection of heat-killed staphylococci into rabbits or guinea-pigs. No antibodies were found when strains which failed to give clumping were injected, and this makes it difficult to account for the high titres in horses injected with toxoid from the Wood 46 strain since this strain does not clump and is apparently free from antigen. High titres were obtained after four to five injections of young cultures of the Newman strain killed by heating at 100° for 5 min. and adjusted to about 4×10^9 cell/ml. As previously found (Duthie, 1954) the opalescent supernatant fluids of partially autolysed washed suspensions when absorbed on $AlPO_4$ were antigenic; and when these supernatant fluids were brought to pH 4·6 and the deposits removed the fluids were equally active both as antigens and as inhibitors of antibody in the antibody inhibition test. Supernatant fluids from cells disintegrated by gentle autolysis with shaking in the cold or by vigorous shaking with ballotini were equally antigenic when absorbed on to $AlPO_4$.

Interaction of fibrinogen and cells

Influence of pH *value.* While clumping of the cells occurred only above pH 5·1, absorption took place over the pH range 2–10. The fibrinogen showed loss in reactivity below pH 4·6 and in consequence in Table 3 much higher fibrinogen concentrations were used below pH 4·6. It will be noted that some absorption occurred at all pH values, as shown by comparison with control solutions not absorbed and by the clumping of the washed and resuspended cells. Absorption increased up to pH 7 and then remained maximal. Clumping of the cells began at pH 5·1 and was maximal at pH 5·5, the isoelectric point of fibrinogen, and above. Clumped cells were readily unclumped by bringing the pH below 5·1 and could be clumped once more by the addition of alkali.

Table 3. *The influence of change in* pH *on absorption of human fibrinogen and on clumping of staphylococcal suspensions*

	Clumping of staphylococcal suspensions						
	Unabsorbed fibrinogen			Absorbed fibrinogen			
	Concentration of fibrinogen absorbed (%, w/v)*						Clumping of cells in fibrinogen 0·02 % (w/v)
pH value	0·02	0·01	0·0005	0·02	0·01	0·0005	
2	±	–	–	+ +	±	–	–
4	+	–	–	+ +	+ ±	–	–
	Concentration of fibrinogen absorbed (%, w/v)*						
	0·002	0·001	0·0005	0·002	0·001	0·0005	
4·6	+ +	+ ±	+	+ +	+ ±	+	–
5·1	+ ±	+	±	+ +	+ ±	+	±
6·0	+ +	+ +	+	+ +	+ +	+ ±	+ +
7·0	+ +	+	–	+ +	+ +	+ ±	+ +
8·0	+	tr.	–	+ +	+ +	+ ±	+ +
9·7	+	–	–	+ +	+ +	+ ±	+ +

* Owing to the denaturation of fibrinogen at acid pH, the concentration of fibrinogen necessary at pH values below 4·5 is about 10 times that for pH values above 4·5.

Effect of Na *and* Ca *ions.* Absorption but not clumping was Na^+ dependent, trace clumping being present in 0·01 M-NaCl and moderate clumping in 0·015 M-NaCl. Fibrinogen absorbed on coated cells was not removed by washing in distilled water or in dilute acetic acid. Cells washed in versene (ethylenediamine-tetra-acetic acid), which is a strong Ca^{++} chelating agent, and subsequently shaken in fibrinogen containing 1% (w/v) versene clumped normally, showing that neither absorption nor clumping was Ca^{++} dependent. Clumping occurred in 20%, but not in 30% urea, provided that sufficient Na ions were present. There was some inhibition of clumping in 3 M-NaCl but not in molar solutions.

Action of antiserum. Antiserum prevented not only absorption of fibrinogen by cells, but also clumping of cells to which fibrinogen was first added followed by antiserum before shaking. On the other hand, no evidence for the converse, i.e. inhibition of antiserum absorption by fibrinogen, was obtained. Thus cells were treated with fibrinogen to which antiserum was then added, both reagents being at limiting concentrations, and were compared with cells in antiserum only. On removing the cells an equivalent amount of fibrinogen was added to these control tubes. Titration of both lots of supernatants with cells did not reveal the difference that would have appeared if there had been impairment of antiserum absorption in the former mixtures. On another occasion antiserum was absorbed by fibrinogen-coated and by washed cells at pH 4·8. No difference was detected in the inhibitory power of the two lots of supernatants when compared at neutral pH. Comparison at higher pH values was impossible because of the clumping of the fibrinogen-coated cells.

Absorption of fibrinogen by streptococci and other particles

Fibrinogen absorption by other bacteria. The following tests were made to determine whether organisms other than coagulase-positive staphylococci could either absorb or clump in human or bovine fibrinogen. Washed bacteria and yeast suspensions at a standard density were shaken in 0·02% (w/v) solutions of the two fibrinogens. When clumping was not seen the cells were washed twice and resuspended in saline, after which an equal volume of a standard suspension of coagulase-positive staphylococci was added and the suspensions shaken once more. Any clumping due to absorption of fibrinogen by the washed bacteria was then noted. Absorption tests were often made with three different concentrations of the fibrinogens and these were found to agree with the results already obtained. Of all bacteria and yeasts examined only streptococci of groups A, C and G absorbed human fibrinogen (Table 4). Neither of two freshly isolated strains of *Streptococcus viridans* from cases of subacute endocarditis nor any strains of *S. faecalis* were positive; seven strains of *S. pneumoniae* were all negative. None of the following organisms showed any ability to absorb fibrinogen or to clump in its presence: *Staphylococcus albus*, *S. citreus*, *Micrococcus tetragenus*, *Neisseria gonorrhoeae*, *N. catarrhalis*, *N. meningitidis*, *Proteus vulgaris*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Candida albicans*.

There was some variation in the behaviour of those streptococci which had absorbed fibrinogen. Several gave fine immediate clumping with fibrinogen which was often more marked on washing and resuspension in saline. Others showed only slight or no clumping until the addition of the coagulase-positive staphylocci when gross floccules appeared instantly on shaking. These fibrinogen-coated streptococci clumped only on the addition of whole cells or particulate matter from crushed cells. They did not clump with thrombin or with free coagulase and prothrombin, nor did they clump with staphylococci in the presence of staphylococcal antiserum. All those streptococci which absorbed fibrinogen did so in amounts comparable to that absorbed by staphylococci, and absorption took place over the whole pH range from 2 to 10. Capsulated streptococci absorbed fibrinogen as readily as did non-capsulated forms.

Table 4. *Absorption of human fibrinogen by streptococci and clumping in saline*

Group ...	A	B	C	D	E	F	G	H	L	M	N
Strains tested	9	9	4	3	4	2	4	2	2	2	2
Strains clumping alone	3	0	0	0	0	0	0	0	0	0	0
Strains clumping with coagulase-positive cells	9	0	4	0	0	0	3	0	0	0	0

Particles. When suspensions of coagulase-positive staphylococci were used to test for the presence or absence of fibrinogen it was possible to show that fibrinogen was absorbed readily by particles of a number of materials, mainly various diatomaceous earths. The conditions for absorption were similar to those obtaining with staphylococci and streptococci inasmuch as it occurred over a wide range of pH values and was independent of the presence of Na^+. It was clear that 'Super cel', 'Hyflo super cel', 'Filter cel' (Johns-Mandville), kieselguhr, kaolin and talc readily absorbed fibrinogen. When washed, the majority of the particles clumped readily when coagulase-positive staphylococci were added and the suspensions shaken. Like streptococci these fibrinogen-coated particles failed to clump with thrombin or with free coagulase + prothrombin.

Tanned erythrocytes. Human erythrocytes were treated with tannic acid and coated with human fibrinogen following the method of Boyden (1951), except that they were resuspended in a saline phosphate buffer without the addition of serum. When 0·04 ml. of a 2·5 % suspension of these fibrinogen-coated cells was added to 0·2 ml. amounts of increasing dilutions of the standard staphylococcal cell suspension and the tubes shaken for a few moments, it was found that there was good clumping at a dilution of 1/20 corresponding to 2×10^8 cells/ml. It was hoped to use this technique and also to use fibrinogen-coated particles of a diatomaceous earth for the assay of the clumping material liberated from the staphylococcal cells; within limits set by the nature of the material this has been possible. Suspensions of 2×10^{10} staphyloccocal cells were partially disintegrated by means of: (*a*) shaking for up to 2 hr. with Ballotini beads; (*b*) treatment for 45 min. with a Mullard low-frequency sonic disintegrator kindly lent by Dr D. D. Woods. Both methods decreased the optical density of the suspensions to about 20 % of the original. Extraction of the cells with saline gave a material which was strongly inhibitory to antibody, but was only weakly active when mixed with either fibrinogen-coated red cells or particles. It was noted that the clumping titre for cells and particles fell with the amount of centrifuging that the preparation had received. Nevertheless, it was clear that these suspensions which contained particles barely visible under the microscope were able to inhibit antibody and to clump fibrinogen when suitably absorbed. They appeared to have no action on fibrinogen solutions. Numerous attempts to dissolve the particles or to remove the material from the surface of the cells by acids or alkalis were unsuccessful. Suspensions which were gently rocked at 4° for prolonged periods released a material which was inhibitory to antiserum and antigenic when absorbed on $AlPO_4$, but this had little or no clumping action on either coated cells or particles. In contrast to fibrinogen-coated streptococci and particles, the fibrinogen-coated erythrocytes were readily clumped by thrombin and by free coagulase + prothrombin mixtures.

Comparison of clumping by fibrinogen and agglutination by antibody. Because of a certain similarity between the clumping of staphylococci by fibrinogen and agglutination caused by antisera the following reaction was examined in some detail. When washed coagulase-positive staphylococci, coated with antisera, were shaken with uncoated staphylococci a fine clumping was seen which resembled that given by human fibrinogen at 10^{-6} (w/v). In practice the standard staphylococcal suspension was treated with antiserum at a final concentration of 30 units/ml. for 10 min. at 37° and was then washed twice and resuspended to its original density. No clumping was seen on shaking, but only when an equal volume of untreated cells was added when clumping followed after a few minutes shaking. The reaction is similar to that described by Heidelberger & Kabat (1934) when clumping of pneumococci was brought about by the addition not only of cells of the same group, but by the group specific polysaccharide. The reaction depends firstly on absorption of the specific antibody to the cell and secondly on the absorbed antibody uniting by free valencies with fresh sites on the non-sensitized cells, thus building up aggregates. It was therefore possible to determine the influence of certain ions on absorption of antibody and on the clumping of normal cells, and to compare this with similar findings when fibrinogen acted on staphylococci, streptococci and diatomaceous earth (Table 5).

The four types of reaction were essentially similar. Absorption of antibody or fibrinogen occurred over the whole range of pH values from 3 to 10 for antibody, and from 2 to 10 for fibrinogen, and was always independent of the Na^+ concentration. Clumping of the mixture of sensitized and non-sensitized cells was independent of Ca^{++}, required a minimum of 0·01 M-Na^+ and took place between pH 4 and 10. Clumping of fibrinogen-coated bacteria and particles also required a minimum of 0·01 M-Na, was independent of Ca and occurred at pH values between 5·1 and 10·0. All reactions were reversible inasmuch as any alteration of the pH value to one outside either extreme or any lowering of the Na+ below 0·01 M, caused unclumping with further clumping when the former values were restored. This was most easily demonstrated with fibrinogen which was more stable to pH changes than was antibody.

Table 5. *Comparison of conditions for absorption and clumping by fibrinogen and staphylococcal cell antibody*

Clumping systems	pH range for antibody or fibrinogen	pH range for clumping	Influence of ions
Antibody coated *Staphylococcus aureus* + normal *S. aureus*	3·0–10·0	4·0–10·0	Na^+ not needed for absorption but at least 0·01 M-NaCl essential for clumping which was reversible outside pH limits given
Fibrinogen + *S. aureus*	2·0–10·0	5·1–10·0	
Fibrinogen coated *Streptococcus haemolyticus* + *Staphylococcus aureus*	2·0–10·0	5·1–10·0	
Fibrinogen coated 'Supercel' + *S. aureus*	2·0–10·0	5·1–10·0	

DISCUSSION

Coagulase-positive staphylococci are distinguished from others by their ability to absorb fibrinogen, to clump, and to form a common antibody which inhibits this phenomenon. The remarkably good correlation observed between the three properties at all times and under all circumstances makes it likely that the same substance is responsible, although this cannot be proved conclusively because of failure to separate a soluble material from the cells. Since the absorption of fibrinogen and the clumping do not appear to be due to the action of an enzyme, it is proposed that the material responsible be called 'clumping factor' rather than bound coagulase as previously suggested. The marked stability of clumping factor is noteworthy; at pH 11 there is partial destruction in 24 hr. at 37° but none at 20°, although treatment at this pH value usually destroys surface antigens in other bacteria. Clumping factor was resistant to boiling and was unaltered by acetone, ethanol or ether extraction. Autoclaving and treatment with certain proteolytic enzyme preparations destroyed it. It was recovered in a particulate form from the supernatant fluids of suspensions of cells disrupted mechanically. It should be noted that such supernatant fluids were active antigenically both by the antibody inhibition test and in producing antibody in animals; but their ability to induce clumping of fibrinogen-coated particles, of red cells, or of antibody-coated staphylococcal cells was limited. Clumping factor might well be a peptide complex, but until it can be obtained in a soluble form which can be demonstrated by a suitable test this cannot be verified.

The clumping of staphylococci by fibrinogen is now seen to occur in two stages. First, there is an absorption of fibrinogen by the cells similar to that found with certain streptococci and with certain inanimate particles, e.g. diatom skeletons. All these absorptions occur over the range pH 2–11, in which fibrinogen is stable, and they are independent of ions. The second stage, clumping, begins at pH 5·1, is maximal at pH 5·5 the isoelectric point of fibrinogen, and continues up to pH 10. In this it resembles the clotting of fibrinogen by thrombin (Lorand, 1954). Since clumping is maximal at the isoelectric point of fibrinogen, i.e. when negatively-charged groups appear on the fibrinogen molecule it may be assumed that the fibrinogen first decreases the mutually repelling electrostatic forces on the staphylococci, as shown by the decrease in surface charge, and that free negatively-charged groups of the absorbed fibrinogen then unite with positively charged groups on other cells, thus giving aggregation. A similar explanation for serum agglutination was put forward by Heidelberger & Kabat (1934).

To account for the factors involved in the clumping of staphylococci and of streptococci the following scheme can serve as a working hypothesis. Staphylococci may be thought of as having primary absorbing sites A and secondary binding sites B which only unite with fibrinogen suitably oriented by first being absorbed on the primary sites. The secondary sites B are presumably absent from streptococci of groups A, C and G which can, however, form clumps when whole staphylococci or particles containing B sites are added to the fibrinogen-coated cells. When antiserum is added to normal staphylococcal cells both primary and secondary sites are blocked by antibody and the cells cannot absorb fibrinogen. When antiserum is added to fibrinogen-coated cells then the secondary sites B are blocked by antibody and the suspensions cannot clump when shaken. The hypothesis covers all the known facts but cannot be proved since it was found impossible to destroy one presumptive site only. From the nature of the experiments it would appear that a single substance is responsible, the sites being different groups.

One of the main uses of staphylococcal clumping factor lies in its ability to detect extremely small amounts of either normal or denatured fibrinogen of certain animals, in solution or when absorbed on particles. Pure human fibrinogen showed visible clumping at 1 mg./l. and human plasma showed visible clumping at a dilution of 1/20,000 when mixed and shaken with an equal volume of the standard bacterial suspension made slightly more sensitive by boiling for a few minutes. Allowing that this plasma contained between 0·2 and 0·4% (w/v) fibrinogen this meant that each cell was clumped by as little as 12–24 molecules of fibrinogen. That most diatomaceous earths readily absorb fibrinogen was well known, but that this property is shared only by pathogenic staphylococci and groups A, C and G streptococci is more surprising. Absorption of fibrinogen by these bacteria is apparently due to the presence in all these organisms of a similar substance which is resistant to moderate heat (boiling but not autoclaving), to moderate pH changes (pH 2–11) and to extraction by lipid solvents. Since it was destroyed easily by proteolytic enzymes it is presumably a peptide complex. Bacteria which failed to absorb fibrinogen could not be made to do so by any chemical or physical means employed.

As mentioned earlier (Duthie, 1954), staphylococci of doubtful pathogenicity are found from time to time which produce free coagulase but fail to clump even in strong concentrations of fibrinogen or plasma. It has now been found that on several subcultures the majority of these strains give clumping though never in high titre. Similarly, strain variants have been found in cultures which give clumping but show no free coagulase either in tube or plate tests. Neither of these variants has yet been found in pathological lesions, and it may be assumed that either the conventional slide or the tube test is a good guide to pathogenicity in material recovered from lesions though not necessarily from body surfaces.

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The Development of the 'Straight Rod Type' of *Lactobacillus bifidus*

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SUMMARY: Microscopical examinations of several *Lactobacillus bifidus* strains have shown that the 'bifid' morphology of the cells, which seems to predominate *in vivo*, is readily changed on subcultivation in different media *in vitro*. At first highly branched and larger forms occur, and from these mycelial branched filaments develop. The mycelial cells may swell, in which case intensely staining coccoid granules can be detected in the cells. Ultimately, the filaments break up, and give rise to the development of regular short straight rods. Obviously these straight rods appear from the coccoid granules.

It is well known that *Lactobacillus bifidus* occurs as two different morphological types: a 'bifid' type occurring as branched cells, and a 'straight rod' type showing regular straight rods (e.g. Norris, Flanders, Tomarelli & György, 1950). In the intestines of young infants the bifid form may predominate, but this morphological character is readily lost *in vitro* with a change to the straight rod type. The change is reported to be irreversible (Norris *et al.* 1950; Norris, De Sipin, Zilliken, Harvey & György, 1954), and probably instantaneous (Norris *et al.* 1950). The change takes place most frequently in complex media containing cow's milk or vegetable extracts. Some authors have succeeded in maintaining their strains unchanged in chemically defined media (e.g. György, 1953; Petuely & Lynau, 1954). Most of the recent studies of *L. bifidus* have dealt with its nutrition, and in this connexion attention has been drawn to the abundance of this bacterium in the intestines of infants fed on human milk. Hence the major interest has been directed to the bifid type, and the morphological variation has been investigated only in attempts to avoid it. Observations made during the routine microscopical control of bifid cultures led the present author to a more extensive investigation of the morphological variation of *L. bifidus*. This seemed to deserve special attention since certain features showed a resemblance to the morphogenesis described earlier as characteristic of some systematically related bacteria, e.g. polymorphic corynebacteria.

METHODS

All the strains investigated were isolated from the faeces of breast-fed infants. Isolation was carried out according to ordinary techniques; single-cell cultures were not investigated. All isolates were obtained from high dilutions (from tubes or plates where only 1–10 colonies had grown). A nutrient agar prepared according to Hassinen, Durbin, Tomarelli & Bernhart (1951) was usually employed for isolation. This medium seems to permit slight growth of *Lacto-*

bacillus bifidus, at least in the isolation passage, but as reported by Hassinen *et al.* (1951), and as found by the present author, other intestinal bacteria may fail to grow in it. An additional spreading of the cultures was carried out, and re-isolates were taken from suitable high dilutions.

The cultures were maintained in liquid media or as stabs in the Hassinen *et al.* nutrient agar. The media used were prepared according to Hassinen *et al.* (1951), György (1953), and Gyllenberg, Rossander & Roine (1953). Tomato agar (Difco, Kulp) was also used for stabs. The stock cultures were renewed once or twice each week.

The smears were stained unfixed with Hucker's crystal violet or Ziehl's carbol fuchsin. Phase-contrast equipment was used for the examination of unstained smears.

RESULTS

The cell shape of primary cultures was quite typical. The rods were short (2–4 μ. long), the ends were pointed or cleft, and curved rods were abundant. Manifold branchings and mycelial forms were not seen. This cell shape (referred to below as the bifid type), which obviously predominates *in vivo*, is illustrated in Pl. 1, fig. 1. On continued cultivation *in vitro*, the original bifid shape disappeared in a few subcultures. The size of the organism increased (4–8 μ. long) and manifold branching was regularly encountered (Pl. 1, fig. 2). Such organisms (referred to as the 'branched' type) were often swollen, especially in old cultures, in which they stained heterogeneously and showed chains of deeply stained granules. When cultivation was continued, still larger branched mycelial filaments are found (Pl. 1, fig. 3). It seems that these forms developed especially when the cultures grew very rapidly.

Cultures with organisms in the branched state were maintained unchanged through several subcultures, although the cell size and the branching frequency were highly variable. Ultimately, however, a rapid change to straight rods took place; these rods were regular and short, and 1–2 μ. long (Pl. 1, fig. 4).

It seemed that the short rods developed from the mycelial filaments. In swollen filaments chains of granules occurred (Pl. 1, fig. 5), as already mentioned. In some cultures, however, the mycelial filaments were abruptly broken up, and only 'granule chains' remained. These chains were often branched, and showed arrangements similar to those of the granules in the original intact filaments (Pl. 1, fig. 6). Figures 5 and 6 of Pl. 1 represent the appearance of samples from the same culture, taken at 20 and 23 hr., respectively, i.e. at an interval of 3 hr. Pl. 1, fig. 7, also illustrates this phenomenon: (*a*) represents a mycelial filament in an early stage of breakdown; (*b*) shows branched chains of short rods. The short straight rods which develop in this way rapidly lose their ability to form chains, and they then occur in irregular masses (cf. Pl. 1, fig. 4). It is possible that the straight rods under certain conditions may become branched or bifid, but the author has not succeeded in obtaining definite evidence on this point.

The observations described above show that the appearance of the straight rods is a result of a gradual development, although the final phase seems to proceed very rapidly. It can be concluded that the tendency to develop branched mycelial filaments characterizes the first step towards the straight rod type, since the straight rods obviously arise from granules which occur in the filaments. The branched filaments are broken up into short rods in a similar way to that described for the transformation of filamentous cells of corynebacteria to coccoid forms (cf. Conn & Dimmick, 1947). It can be supposed, therefore, that the granules in *Lactobacillus bifidus* correspond to the beads or 'arthrospores' which occur in certain corynebacteria. Should this prove to be the case, the relationship of *L. bifidus* to the corynebacteria, which, as stated by some authors (e.g. Olsen, 1949), may be a very close one, needs further explanation.

Primary bifid *Lactobacillus bifidus* cultures require good anaerobic conditions for growth, whereas the branched cultures are microaerophilic, and the straight rod cultures are usually capable of aerobic growth. Accordingly, it seems possible that the first morphological change (from bifid to branched) may be induced under conditions where the oxidation-reduction potential is not low enough to permit the bifid type to maintain its characteristic behaviour. Petuely & Lynau (1954) reported that *L. bifidus* can be maintained in the bifid state when the media are richly supplied with ascorbic acid. According to these authors, other cell shapes may indicate that degeneration is taking place under unfavourable conditions. It seems more probable, however, that these changes are due to true morphogenesis, since they take place under conditions where the generating new types seem to grow vigorously.

Single-cell cultures were not studied in the present investigation. This fact may introduce some measure of uncertainty. However, the phenomena described were observed with several different cultures, and as pointed out earlier, it may be assumed that the cultures studied were pure.

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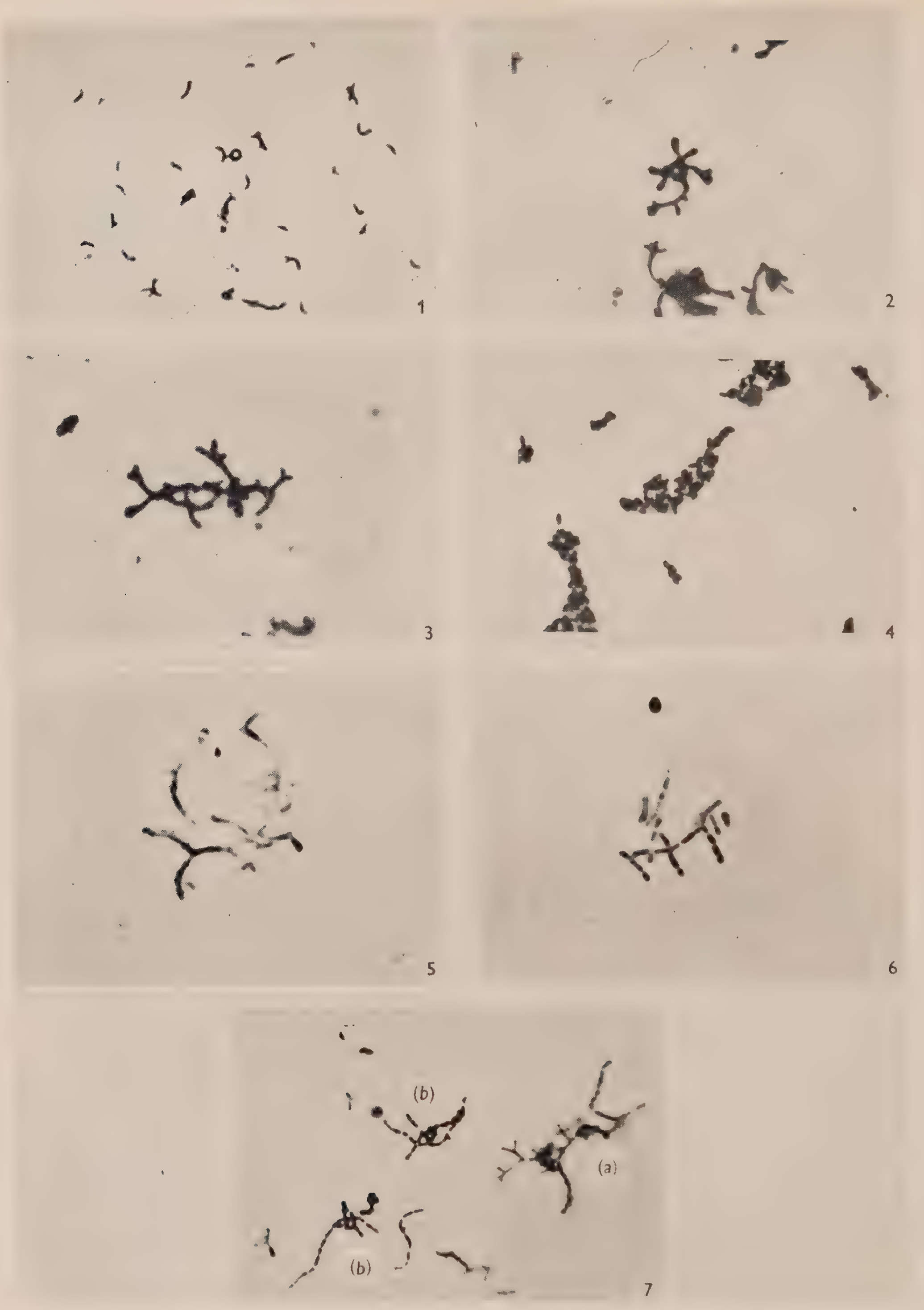

H. G. GYLLENBERG—MORPHOLOGY OF *LACTOBACILLUS BIFIDUS*. PLATE 1

(*Facing p.* 397)

EXPLANATION OF PLATE

Fig. 1. Primary agar shake culture. Medium according to Hassinen *et al.* Age: 72 hr.; × 1000; crystal violet.

Fig. 2. Subcultured *in vitro* for 6 weeks. Tomato agar stab. Age: 72 hr.; × 1000; crystal violet.

Fig. 3. Subcultured *in vitro* for 10 weeks. Liquid medium according to György. Age: 48 hr.; × 1000; carbol fuchsin.

Fig. 4. Subcultured *in vitro* for 10 weeks. Tomato agar stab. Age: 48 hr.; × 1000; carbol fuchsin.

Fig. 5. Subcultured *in vitro* for 12 weeks. Liquid medium according to Gyllenberg *et al.* Age 20 hr.; × 1000; phase contrast.

Fig. 6. Subcultured *in vitro* for 12 weeks. Liquid medium according to Gyllenberg *et al.* Age 23 hr.; × 1000; phase contrast.

Fig. 7. Subcultured *in vitro* for 10 weeks. Tomato agar stab. Age 48 hr.; × 1000; carbol fuchsin. (*a*) mycelial filament in an early stage of breakdown; (*b*) branched chains of short rods.

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Further Nutritional Studies of *Venturia inaequalis*

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SUMMARY: A series of nutritional experiments was carried out on cultures from single ascospores isolated from a single ascus of *Venturia inaequalis.* The results showed: (*a*) thiamine stimulated all cultures and other vitamins did so to a much less extent; (*b*) decoctions of apple leaves contained stimulatory substances; (*c*) these substances were amino acids, chiefly isoleucine, glutamine, serine and phenylalanine; (*d*) best growth was given by some cultures in the presence of thiamine and various amino acids; (*e*) the reaction of the fungus cultures to the decoctions, amino acids and thiamine varied according to the cultures and the combination of nutrients. There were indications that the nutritional requirements reflected the genetic composition of a given culture; (*f*) in the absence of leaf decoctions the best sources of nitrogen were Oxoid peptone and asparagine, and growth with these substances reflected the genetic composition of the organism; (*g*) at least ten amino acids were shown to be present in the leaves of all or some apple varieties.

In a previous paper Fothergill & Ashcroft (1955) showed that a strain of *Venturia inaequalis,* the cause of apple scab, obtained from the Commonwealth Mycological Institute, gave maximal growth when grown at 20° for 30 days at pH 5·8 in a balanced solution containing: 4% (w/v) glucose; 0·004 M-K_2HPO_4; 0·002 M-$MgSO_4.7H_2O$; 0·0375 M-NH_4NO_3; Zn and Mn at 2 p.p.m.; sufficient amounts of thiamine, pyridoxine, folic acid, nicotinic acid and ascorbic acid. Growth without these growth factors was negligible, but thiamine alone gave the greatest stimulation. Suggestions were made by Keitt and co-workers (Keitt, Palmiter & Langford, 1938; Keitt & Langford, 1941; Keitt, Langford & Shax, 1943; Leben & Keitt, 1948) that a genetic factor, or factors, in *V. inaequalis* which was responsible for pathogenicity might be related to the nutritional requirements of the given pathogenic strain. In the present communication the results of a study of cultures from single ascopores are given.

METHODS

Eight cultures grown from eight ascospores isolated from a single ascus of *Venturia inaequalis* were kindly supplied by Professor G. W. Keitt, Pathology Department, Wisconsin University. They were numbered according to the position of the ascospore in the ascus, culture 1 having been derived from the apical spore and the others in order downwards. The following are known to be chromatid mates: 1, 2; 3, 4; 5, 6; and 7, 8. Single spore cultures were made from each of these, but unfortunately culture 7 died in transit. These cultures are designated C. 1, C. 2, ..., C. 8. That part of the medium given above which contained the mineral salts and glucose only will be subsequently referred to as the basal medium. In each experiment 30 ml. volumes of basal medium were used in 150 ml. Erlenmeyer flasks with five replicates for each variation of medium; sterile phosphate solutions were added to the flasks after autoclaving. The inoculum for all experiments was prepared by adding 15 ml. sterile distilled water to a $6 \times \frac{3}{4}$ in. tube containing a well-grown culture on agar (30 days old). The surface of the agar was scratched with a sterile needle and 1 ml. of the resultant mycelial suspension was used as inoculum for each experimental flask of liquid medium. It was found by experience that the exact amount of the inoculum did not appreciably affect growth. Cultures were incubated at 20° for 30 days at pH 5·8, and after incubation the mycelial felts were harvested, dried at 95° and weighed, amounts being expressed as mg. dry wt./flask.

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Apple leaf decoctions were made by gently boiling 25 g. air-dried apple leaves for 30 min. in distilled water. After filtering, the amount of decoction was made up to one litre and incorporated into the basal medium so that each culture flask contained 5 ml. decoction. Decoctions were made from the following apple varieties: Laxton's Superb, Bramley's Seedling, Newton Wonder, James Grieve, Cox's Orange Pippin, Blenheim Orange Pippin, White Transparent, Wealthy.

RESULTS

Effects of apple leaf decoctions on growth

It is known that apple varieties respond differently to different strains of *Venturia inaequalis*. This observation may perhaps be correlated with differences in the food requirements which the apple is able to supply to the parasite. The growth of the monoascosporal cultures in the basal medium with the addition of decoctions of leaves from the different apple varieties was tested. The seven single cultures were each grown with each mixture of decoction + basal medium in five replicates; there was also a control with basal medium alone. The results are given in Table 1.

Table 1. *Growth response of monoascosporal cultures of* Venturia inaequalis *to media containing leaf decoctions of different apple varieties*

Results given as nearest mg. dry weight.

	Basal medium + decoctions of								
Culture	Laxton's Superb	Bramley's Seedling	Newton Wonder	James Grieve	Cox's Orange Pippin	Blenheim Orange Pippin	White Transparent	Wealthy	Control no decoctio
C. 1	159	147	67	36	12	61	104	62	15
C. 2	95	68	55	32	38	80	34	39	11
C. 3	49	78	101	51	138	176	106	45	20
C. 4	38	44	46	44	63	62	43	43	16
C. 5	182	140	147	217	150	423	167	37	7
C. 6	74	98	182	155	134	258	125	62	9
C. 8	72	73	125	122	167	116	104	53	11

With one exception it was seen that all growths with decoction were greater than the growths without decoction. Each culture varied in its amount of growth with each decoction, and with any one decoction the cultures varied in the amount of growth. Generally the faster growing cultures, which are C. 5, C. 6 and C. 8, gave greater mean weights than the slower growing cultures. Notable exceptions are seen in the growth of C. 1 with Cox's Orange Pippin and C. 3 with James Grieve and Wealthy decoctions. Except for C. 1 with Cox's Orange Pippin, C. 2 with James Grieve and White Transparent and C. 4 with Laxton's Superb decoctions, the growth of all cultures with Wealthy decoctions was poorer than with the others. C. 4 is a slow growing culture and its growth with decoction was generally lower than that of the other cultures. Johnstone (1931) found that leaf extracts derived from resistant varieties of apple were more toxic to *Venturia inaequalis* than extracts from susceptible varieties. Thus the presence of toxic substances in the leaves might account for the low growths of some of the cultures. This set of experiments indicated that the apple leaves contained substances stimulatory to the good growth of *V. inaequalis* on the basal medium used.

It was also noted that, in general, cultures C. 1, C. 3 and C. 5 gave greater growth than their corresponding chromatid mates. This result supports that of Keitt & Langford (1941), who found that several apple varieties differed similarly in their resistance to the eight ascosporal cultures of a single ascus.

The nutritional requirements of the monoascosporal cultures

Because of the varying behaviour of the cultures to the different decoctions it became necessary to study the nutritional requirements of the seven cultures C. 1 to C. 8.

The effect of added growth factors and apple leaf decoctions. In this experiment the five growth factors known to stimulate an English strain of *Venturia inaequalis* were used with or without added leaf decoction. A preliminary experiment showed that these five growth factors either did not stimulate growth of the cultures C. 1 to C. 8, or did so only to a limited extent; only cultures C. 2 and C. 3 showed a significant response to thiamine or folic acid. If any of the five growth factors tested was essential to the growth of the fungus in addition to those added in the decoction, addition of it to the basal medium + decoction should be reflected in an increased yield of growth. The growths of cultures C. 5, C. 6 and C. 8 did show a significant increase.

The effect of manganese and β-indolylacetic acid. Some claims have been made that β-indolylacetic acid, the natural growth hormone, increases the growth of fungi in cultures (Hawker, 1950). Epstein & Lilleland (1942) found that apple leaves contained a high proportion of manganese, namely 81 p.p.m. of dry weight. It was possible that *Venturia inaequalis* required a much higher concentration of this metal than hitherto used in our cultures. The effect of 1 p.p.m. of β-indolylacetic acid and 80 p.p.m. manganese was tested with culture C. 5 with and without Bramley's Seedling decoction. The results showed that in the absence of apple leaf decoction neither β-indolylacetic acid nor manganese increased the growth of culture C. 5, but in the presence of decoction both of these substances increased the growth.

The effect of the nitrogen source. Experiments with the Commonwealth Mycological Institute strain of *Venturia inaequalis* CMI 24,006 showed that NH_4NO_3 in the basal medium was a better source of nitrogen than asparagine, provided that the five growth factors were also present. Palmiter (1934) found that the nitrogen source had a marked effect on the growth of *V. inaequalis*. Hence a series of experiments was made to test the effects of different nitrogen compounds on the monoascosporal cultures. The basal medium was used under the usual conditions, but the NH_4NO_3 in it was replaced in turn by $Ca(NO_3)_2$, KNO_3, $(NH_4)_2SO_4$, asparagine and Oxoid peptone in concentrations to give an amount of nitrogen equal to that in 0·0375 M-NH_4NO_3. Each nitrogen compound was tested in the presence or absence of Bramley's Seedling apple leaf decoction. The results are given in Table 2. It has already been noted that

Table 2. *The response of monoascosporal cultures of* Venturia inaequalis *to different nitrogen sources in the basal medium*

Results given as mean of five replicates to nearest mg.

Nitrogen source (≡0·0375 M-NH_4NO_3)

	$Ca(NO_3)_2$		KNO_3		NH_4NO_3		$(NH_4)_2SO_4$		Asparagine		Oxoid peptone	
Culture	+	−	+	−	+	−	+	−	+	−	+	−
C. 1	190	46	114	16	147	15	83	15	185	43	186	328
C. 2	168	9	46	9	68	11	66	4	67	24	63	39
C. 3	91	7	49	12	78	20	129	8	106	36	70	53
C. 4	54	2	43	5	44	16	75	9	62	23	79	36
C. 5	100	3	107	9	140	9	136	14	214	18	307	195
C. 6	90	11	77	6	98	9	145	17	118	14	305	120
C. 8	91	12	67	8	74	11	130	16	238	43	233	108

+ = Bramley's Seedling apple leaf decoction present; − = decoction absent.

apple leaf decoction seems to supply some factor essential for good growth of *V. inaequalis*. Table 2 shows that in the absence of the decoction variation in the inorganic nitrogen source had little effect except in the case of culture C. 1 with $Ca(NO_3)_2$. In the presence of decoction there was a great variation in the responses of the various cultures to the different inorganic nitrogen sources. For example, $Ca(NO_3)_2$ is the best source for C. 1 and C. 2, NH_4NO_3 for C. 5 and $(NH_4)_2SO_4$ for C. 3, C. 4, C. 6 and C. 8. Many of these cultures fell into their chromatid pairs in their reaction to these compounds; the same nitrogen source produced the highest growth in each member of a chromatid pair. Table 2 shows that, in the absence of decoction, asparagine gave a better growth in more of the cultures than the inorganic sources. Oxoid peptone, however, in the absence of decoction gave as good growth as NH_4NO_3 did in the presence of decoction for cultures C. 1, C. 5, C. 6 and C. 8. The growth of culture C. 1 with Oxoid peptone was extraordinarily good, but this strain is fast growing, and its mycelial weight in one medium should only be compared with the corresponding weights in other media, rather than with weights of other strains. In the presence of apple decoction asparagine and Oxoid peptone were both good nitrogen sources for most of the cultures; peptone gave a very high weight of organism with some of the cultures, e.g. C. 5, C. 6 and C.8. It was concluded that the Oxoid peptone contained material which stimulated the growth of *V. inaequalis* and which was possibly present in the apple leaf decoctions.

Determination of the unknown stimulatory material in apple leaf decoction. A quantity of crushed air-dried Bramley's Seedling apple leaves equivalent to 25 g. leaves/l. medium were extracted in a Soxhlet apparatus with ether for 30 hr. The powdered leaves were dried, a decoction prepared and added to the basal medium. This mixture was tested with all the monoascosporal cultures (five replicates) and gave mycelial weights as follows: C. 1...C. 8, 172, 65, 57, 40, 202, 115 and 83 mg. respectively. With all the cultures on this mixture, except C. 3, the results were as good as, or better than, those obtained when a decoction of untreated leaves and NH_4NO_3 in the medium were used. The material in the ether extract added to the basal medium caused no significant increase in growth of the cultures tested. The material in the leaves which increased growth seemed to be water soluble and probably ether insoluble.

A quantity of Bramley's Seedling apple leaves equivalent to 25 g. leaves/l. medium were extracted in cold water in the dark for 3 days and filtered. Of this filtrate one part was tested with culture C. 1 with and without basal medium and gave weights of 174 and 17 mg. respectively. The other part of this filtrate was added to a volume of methylated ether, shaken and separated. The water separation was then steamed and tested with culture C. 1 with and without addition of basal medium; the weights of organism were 167 and 16 mg. respectively. The methylated ether separation was evaporated to dryness, the residue taken up with water and tested with culture C. 1 with and without addition of basal medium; the weights of organism were 34 and 2 mg. respectively.

Thus the growths produced by the cold water extract and the water fraction after ether extraction were about five times that produced by the ether fraction, and they were also slightly greater than the growth produced by the hot-water extract in the previous experiment. Thus the growth stimulatory substances in apple leaves are soluble in cold water and are possibly partially destroyed by heating. The flasks with each nutrient minus the basal medium showed that any carbohydrates and salts present in the decoctions did not influence the dry weight to any appreciable extent; with the ether fraction growth was negligible.

Smith & Jones (1948) determined that the non-volatile compounds insoluble in ether from such leaf extracts as above are carbohydrates, polyhydroxy compounds, salts of organic bases with mineral acids, metallic salts, compounds with sulphonic acid group and amino acids. Our experiments showed that the stimulatory substances present in apple leaves are not carbohydrates and salts. Moreover, carbohydrates are not present in Oxoid peptone, and we have shown that this peptone contains the stimulatory substances. Leben & Keitt (1948) found that the growth of *Venturia inaequalis* on a defined medium was greatly increased by the addition of extracts of malt, liver, yeast or dead apple leaves. They also found that malt extract ash and minor elements did not increase growth, and concluded that the stimulatory substances must be organic materials such as vitamins. Amino acids and various vitamins are recognized growth factors for many fungi and bacteria. The B vitamins are ether insoluble and water soluble, and as chemicals some of them come into the third group mentioned above. Thus it would seem that amino acids, most of which are readily soluble in water, and possibly some vitamins of the B group, are the substances in apple leaves which stimulate the growth of the apple scab fungus.

Leben & Keitt (1948) found thiamine was the only growth factor which stimulated *Venturia inaequalis*; the effect was varied with different cultures. It was shown (Fothergill & Ashcroft, 1955) that a strain of *V. inaequalis* was stimulated in growth by thiamine, folic acid, pyridoxine, nicotinic acid and ascorbic acid. Thiamine alone gave the greatest stimulation. Earlier we showed that the growth of some of the cultures was stimulated by addition of a mixture of these five vitamins + leaf decoction. The results were variable, and it was difficult to select any one growth factor as the one giving the greatest effect; thiamine may have been stimulatory. The effect of thiamine alone and with leaf decoction was next tried on the monoascosporal cultures and on the Commonwealth Mycological Institute strain (C.M.I.). The results are given in Table 3.

Table 3. *The response of monoascosporal cultures of* Venturia inaequalis *and C.M.I. strain to thiamine and leaf decoction*

Results given as mean of five replicates to nearest mg. Control: average value for basal medium alone = 13 mg.

Culture	Basal medium + thiamine	Basal medium + decoction	Basal medium + thiamine + decoction
C. 1	118	147	222
C. 2	30	65	69
C. 3	69	68	70
C. 4	31	64	63
C. 5	62	162	172
C. 6	50	156	203
C. 8	54	196	200
C.M.I.	35	120	160

These results showed that the addition of thiamine to the basal medium greatly increased the growth of all the cultures but to a varying extent. In the previous experiment with five growth factors the effect of the other four possibly masked and interfered with the action of thiamine. In this experiment the addition of thiamine to the medium containing decoction produced a greater growth than the medium with decoction but without the growth factor, varying in amount with the culture used. Strains C. 3 and C. 4 were not affected. In most of the cultures with the decoction + thiamine the growth was as great as, or greater than, the weight obtained in the previous experiment when five growth factors together were added. Thus it seems that thiamine is the chief vitamin which stimulates the growth of the monoascosporal cultures and the remaining four (i.e. pyridoxine, folic acid, nicotinic acid and ascorbic acid) have only a slight effect.

The effect of amino acids on growth of Venturia inaequalis

In the experiments described below the amino acids present in apple leaf decoction were determined by paper chromatography, and then the effects of these compounds with or without thiamine were tried on the cultures. The technique of paper partition chromatography as used by Consden, Gordon & Martin (1944) was used with sheets of Whatman no. 1 filter-paper, size $18\frac{1}{4} \times 22\frac{1}{2}$ in. One- or two-dimensional chromatograms were made with phenol/water and butanol/acetic acid mixture as solvents. The results are aggregated in Table 4.

Table 4. *Amino acid content of leaves of different apple varieties*

Amino acid	Laxton's Superb	Bramley's Seedling	James Grieve	Newton Wonder	Blenheim Orange	Cox's Orange Pippin
Aspartic acid	+	+	+	+	+	+
Glutamic acid	+	P	−	+	P	P
Serine	+	+	+	+	+	+
Asparagine	+	+	−	−	−	−
Alanine	+	+	+	+	+	+
Valine	+	+	+	+	+	+
Phenylalanine	+	+	+	−	−	−
Isoleucine	+	P	−	−	−	−
Glycine	+	P	+	−	P	−
Glutamine	P	P	P	−	−	−

+ = amino acid present; − = acid absent; P = probably present.

Aspartic acid, serine, alanine and valine were detected in the decoctions from the leaves of all the apple varieties analysed. Glutamic acid, phenylalanine, glycine, isoleucine, asparagine and glutamine were detected only in some varieties. Either leucine or isoleucine was present in Laxton's Superb and Bramley's Seedling leaves. Leucine and isoleucine are difficult to separate in chromatograms, but Hulme & Arthrington (1950) stated that isoleucine and not leucine was present in Bramley's Seedling fruit.

A series of experiments was set up using the amino acids found in the apple leaf decoction and the monoascosporal cultures. A test experiment indicated that most information would be obtained by adding the various amino acids separately to the medium in the presence and absence of thiamine, for some amino acids seemed to stimulate growth and others to inhibit it. Various amino acids at 200 mg./l. were tested separately (in quadruplicate).

The results of this set of experiments showed that, except for strain C. 3, where an increased growth was shown, aspartic acid did not affect the growth of the cultures of *Venturia inaequalis* in the absence of thiamine, but in its presence the growth of C. 1, C. 2, C. 5, C. 6 and C. 8 was decreased in spite of the fact that when tested in decoction asparagine increased growth. Glutamic acid did not stimulate the growth of any of the cultures either in the presence or absence of thiamine. Serine increased the growth of C. 6 and C. 8 in the presence and absence of thiamine, and C. 3 only in the presence of the growth factor. All cultures except C. 1 showed an increase in growth with alanine in the presence of thiamine, while C. 5, C. 6 and C. 8 showed increase also in its absence. In the presence of thiamine, phenylalanine increased the growth of C. 3, C. 4, C. 6 and C. 8, but in its absence C. 1, C. 4, C. 6 and C. 8 were stimulated. Isoleucine increased growth of C. 1 and C. 6 and C. 8 only slightly in the absence of thiamine and C. 1, C. 3, C. 5, C. 6 and C. 8 in its presence. Glycine stimulated all cultures in the presence of this growth factor, but only C. 1, C. 5, C. 6 and C. 8 in its absence. In the absence of thiamine valine increased the growth of all cultures to some extent and in its presence that of C. 1, C. 2, C. 5, C. 6 and C. 8. Finally glutamine increased the growth of C. 1, C. 2, C. 5, C. 6 and C. 8 in the presence and absence of thiamine.

DISCUSSION

The experiment with leaf decoctions of various named varieties of apple indicates that apple leaves contain substances which are needed as nutrients for the growth of *Venturia inaequalis* on a medium containing: 4% (w/v) glucose; 0·004 M-K_2PO_4; 0·002 M-$MgSO_4.7H_2O$; 0·0375 M-NH_4NO_3; Zn and Mn at 2 p.p.m. By using cultures derived from the products of a single ascus, which normally behave alike in genetic or chromatid pairs, it was found that in general one set of genetic mates responded differently in growth on the defined medium from the other set. Similarly, in general the cultures differed in their ability to use different nitrogen sources. The chromatid mates of each genetic pair behaved similarly with regard to the nitrogen compound in the presence of apple leaf decoction in the basal medium. In the absence of apple leaf decoction Oxoid peptone and, to a lesser extent, asparagine are good sources of nitrogen. Further experiments showed that in the presence of thiamine the amino acids isoleucine, glutamine, serine and phenylalanine have a differential effect on the growth of the cultures. Thus, although the results are not conclusive, they indicate that nutrition may be genetically controlled in a way which is similar to the inheritance of pathogenicity of *V. inaequalis* (Keitt & Langford, 1941).

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A Multipoint Inoculator for Plating Bacteria or Yeasts

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SUMMARY: A multipoint inoculator has been designed for inoculating agar plates with a maximum of twenty-four strains of bacteria or yeasts simultaneously.

In a previous paper (Beech & Carr, 1955) describing the effect of inhibitory compounds on bacteria and yeasts it was found necessary to make approximately 15,000 separate inoculations. This was time-consuming, and since each plate had to be opened twenty-four times the chances of aerial contamination were considerable. The same problem arises when determining the utilization of sugars, etc., by yeasts, with the auxanographic plate method. These difficulties may be overcome by the simultaneous inoculation of the plate with the required number of organisms.

Earlier references to such methods have been a multiple-point needle (Garrett, 1946) for inoculating an agar plate with plaques of mould colonies and a replica plating method using circles of velveteen (Lederberg & Lederberg, 1952). Garrett's instrument was not ideal for bacteria and yeasts. In the second method each velveteen circle can only be used for approximately five plates since the inoculum size diminishes with each transfer. Further, this method requires plates inoculated in a pre-arranged pattern as a source of the test organisms. It was considered that a modification of Garrett's instrument with the use of liquid inocula would be more suitable for bacteria and yeasts.

The complete apparatus, illustrated on Pl. 1, figs. 1–3, consists of an inoculum tube holder, a multipoint inoculator and a Petri dish holder. Stainless steel, FMB grade, was used for all metal parts, although other non-corrodible metals could be substituted.

Inoculum tube holder. This is a circular metal base plate drilled with twenty-four equally spaced holes, each capable of holding a 9 × 50 mm. Durham tube. On top of this is a block of smaller diameter consisting of two circular metal disks interleaved with heat-resisting rubber, all drilled in the same pattern as the base plate and held in position with screws; two vertical guides are also attached to the base plate. The holes in the rubber sheet are slightly smaller than those in the metal disks and serve to hold the Durham tubes tightly in position.

Inoculator. This consists of twenty-four vertical metal rods, $\frac{1}{8}$ in. diameter with truncated ends which prevent the inocula dripping during transfer. The ods are tapped into the inner basal surface of an inverted metal cup and are positioned to correspond with the centres of the twenty-four holes. The cup is grooved externally to engage with the vertical guides of the inoculum tube holder, and is surmounted by a heat-resistant, insulated handle.

Petri dish holder. This, like the inoculum tube holder, has a base plate and two vertical guides, but the base is recessed slightly to accommodate a Petri dish.

Inoculation procedure

Durham tubes are inserted in the tube holder and covered with aluminium caps (Oxo Ltd., Medical Dept., Thames House, London, E.C. 4). The complete holder and multipoint inoculator are each wrapped in brown paper and sterilized by autoclaving at 15 lb./sq.in. for 15 min. A covered Petri dish containing 20 ml. sterile nutrient agar is placed on the Petri dish holder. The tube holder is unwrapped and the caps covered with an inverted sterile glass dish: each cap is discarded in turn after the addition of 1 ml. inoculum to the corresponding tube. The inoculator is unwrapped, the glass dish removed and held in one hand, while the inoculator is pushed down the guides into the tubes of inoculum. The inoculator is then withdrawn and the tubes re-covered. The lid of the Petri dish is removed with one hand, and the inoculator pushed down the guides of the inoculum tube holder. Set screws are fitted into the top surfaces of the guides on the Petri dish holder and are adjusted beforehand so that when they bear on the base of the handle, the inoculating rods just touch the agar surface. The inoculator is removed and the Petri dish cover replaced. A fresh plate is then placed on the holder and the cycle repeated. In this way a plate may be inoculated simultaneously with twenty-four organisms in 30 sec. compared with 8 min. required for inoculating them singly with a wire loop (Pl. 2, figs. 4, 5). Since the inoculator is bilaterally symmetrical it is advisable to mark one of the grooves and one guide on each holder with cellulose paint. A mark should also be made on the outside of the Petri dish.

To prevent the drops of inoculum from coalescing on the agar plate it is essential to dry the surface of the medium before inoculation, to leave the inoculated plates on the work bench until the drops have dried and, most important, not to add surface-active agents, e.g. Tween-80, to the medium.

Size of spot. To estimate the volume of inoculum deposited by each rod, 1 ml. 20 % NaCl was added to each inoculum tube and the rod holder pressed home. The drops of NaCl solution were transferred to a dried plate of 2 % washed agar using the procedure outlined previously. Twenty-four circles of agar, each with a drop in the centre, were cut out with a cork borer and placed in individual 100 ml. conical flasks. Two other plates were each inoculated six times with the same NaCl solution for comparison but using a conventional 4 mm. wire loop. Comparable circles of agar, without NaCl, were also removed as controls. 10 ml. 0·9 N-HNO_3 was added to each flask, heated to dissolve the agar, cooled and the amount of chloride present estimated by an adaptation of Volhard's volumetric method for chlorides (Vogel, 1939). From this data the volumes of the chloride spots could be calculated.

The average volume deposited by each rod was 0·01 ml. with a variation of ±25%. By comparison spots from the 4 mm. platinum loop were 0·005 ml. with a variation of ±25%. Both methods showed some discrepancies; thus the size of the spot deposited by the rods could be varied by the speed with which they were withdrawn from the inoculum holder. The wire loop suffered from the same defect, and in addition the volume deposited was increased by pressing the loop more heavily on to the agar.

On the basis of these observations the multipoint inoculator, at its present stage of development, is no less subject to variation than the wire loop but it has the advantage of greater speed in operation. It is suitable, therefore, for rapid surveys of a semi-quantitative nature.

We wish to thank Miss M. E. Kieser, B.Sc., A.R.I.C., for adapting the chloride method, Mr D. J. Sissons for the chemical determinations and Mr G. H. Jones, A.R.P.S., for taking the photographs illustrating this article.

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EXPLANATION OF PLATES

Plate 1

Fig. 1. Inoculum tube holder ready for use.
Fig. 2. Oblique view of the inoculator.
Fig. 3. Petri dish holder with uninoculated plate in position.

Plate 2

Fig. 4. Petri dish showing 23 colonies of three different species of lactic acid bacteria inoculated simultaneously.
Fig. 5. As Fig. 1, showing 24 colonies of three different species of yeasts inoculated simultaneously.

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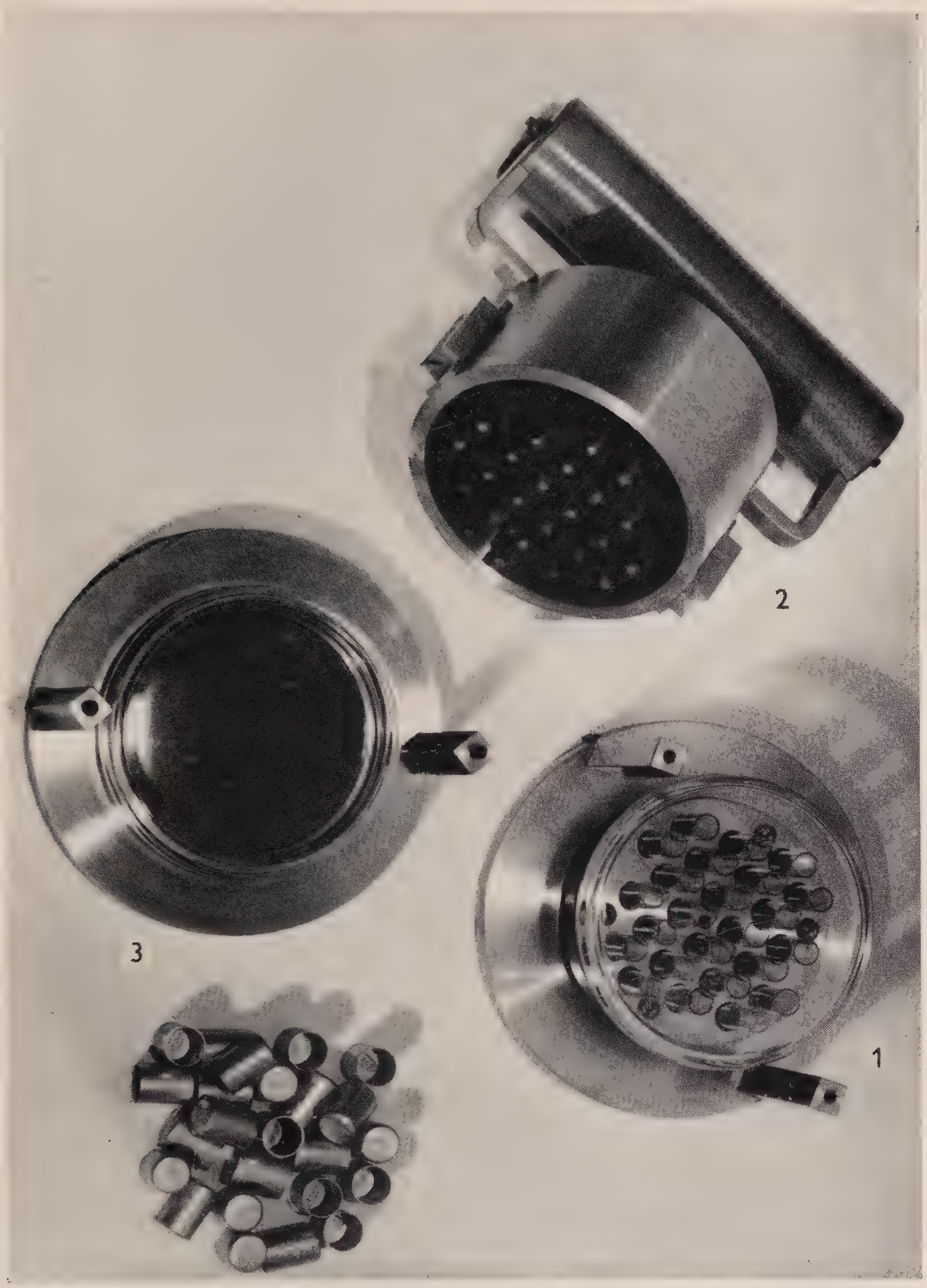

F. W. Beech, J. G. Carr & R. C. Codner – A multipoint inoculator. Plate 1

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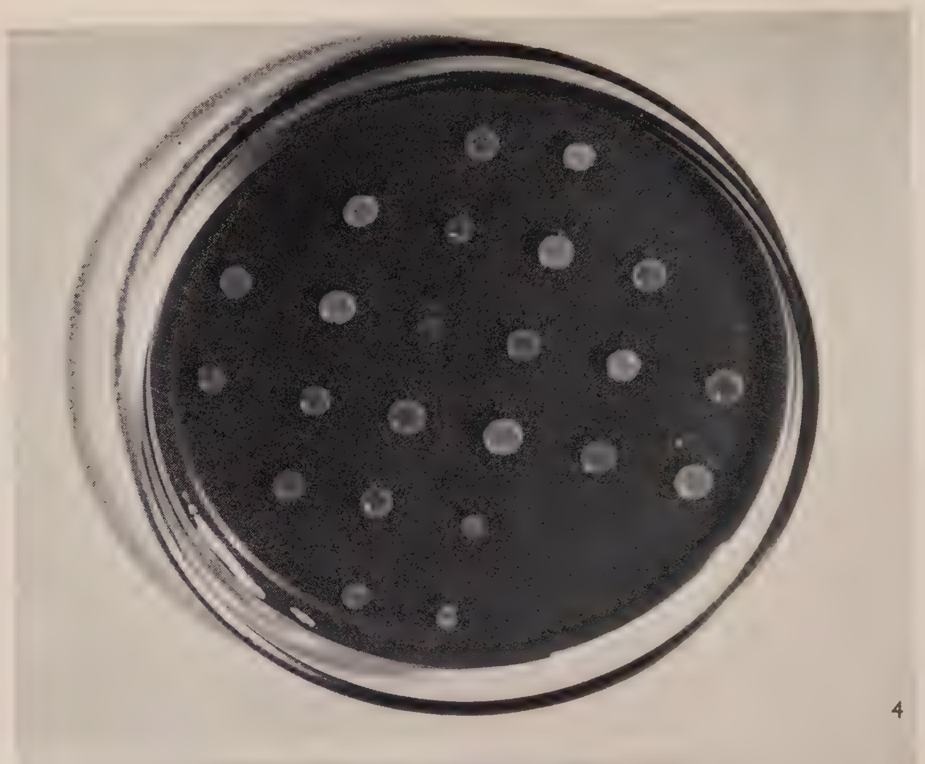

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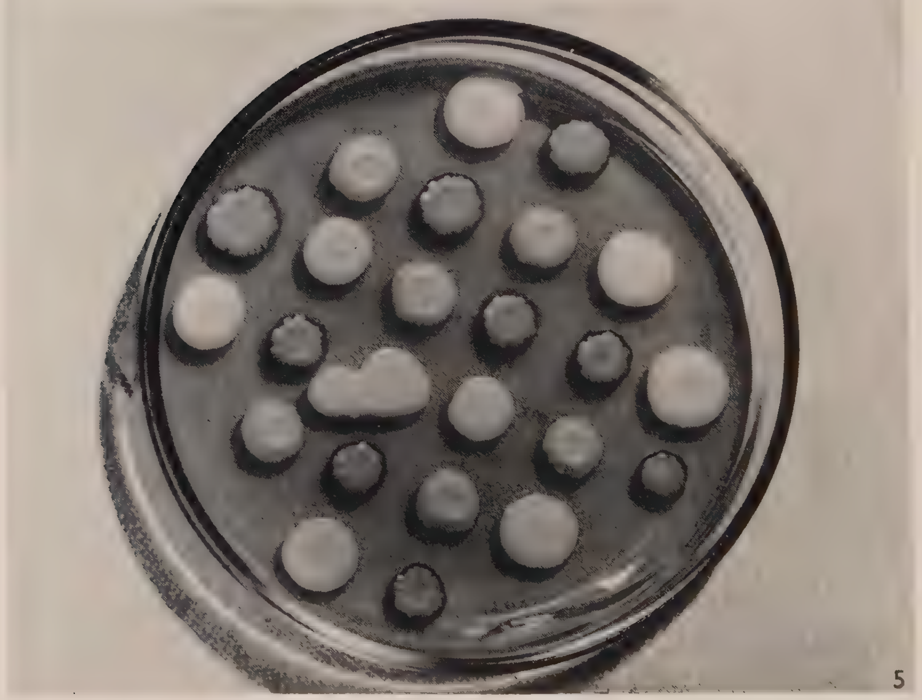

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F. W. Beech, J. G. Carr & R. C. Codner—A multipoint inoculator. Plate 2

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The Cytology of Bacteroids from Root Nodules of Subterranean Clover (*Trifolium subterraneum* L.)

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SUMMARY: Bacteroids from effective nodules of subterranean clover were ovoid, pear-shaped, or slightly branched and measured $5\text{–}7 \times 4\text{–}6\,\mu$. In the fully developed bacteroid the nuclear material was central, divided into discrete bodies and showed signs of degeneration. Surrounding the nucleus was a perinuclear region, probably derived from it, and this was surrounded by cytoplasm which contained several mitochondria-like granules. The development of bacteroids from rod-shaped cells is described. Bacteroids from the ineffective nodules studied possessed no perinuclear region, and, in contrast to those from effective nodules, accumulated varying amounts of glycogen, depending on the length of time for which the bacteroids survived in the host cell.

The symbiotic nitrogen-fixing nodule bacteria have been the subject of much research, and it is surprising that the structure of the organisms within the host cells has not received more attention. The term 'bacteroid' has been given to the swollen irregularly shaped bacteria found in the cytoplasm of infected host cells in nodules on legume roots. Morck (1891) made a careful study of bacteroids, and his drawings show cells with clear central areas surrounded by granular cytoplasm. Uher (1937) described spindle- and globiform-shaped nuclei in bacteroids from *Trifolium* species, and Schaede (1939, 1941) demonstrated a diffuse Feulgen-positive reaction in bacteroids from *Lupinus* and *Neptunia* nodules but not in those from *Vicia faba*. Baylor, Appleman, Sears & Clark (1945) described nuclear granules visible in electron micrographs of nodule bacteria from cultures and excised nodules, and Bisset (1952) included some observations on bacteroids in a paper on life cycles in *Rhizobium* species. The reviews of Fred, Baldwin & McCoy (1932) and of Allen & Allen (1950) give further references to previous work on this subject.

As with other legume species, root nodules on subterranean clover may not always benefit the host plant by fixing atmospheric nitrogen, in which case they are termed 'ineffective'. In this paper, work is described in which bacteroids from both 'effective' and 'ineffective' nodules were examined by a variety of cytological techniques.

METHODS

Organisms. Subterranean clover (*Trifolium subterraneum* L.) variety Mt. Barker was used throughout as host plant. Plants were grown in test tubes on a nitrogen-free agar medium according to the method of Thornton (1930). After germination each seedling was inoculated with one of the strains of *Rhizobium trifolii* listed in Table 1, by means of one drop of a suspension from a yeast water agar culture. The plants were kept in a glasshouse and nodules were examined at various intervals. Both effective and ineffective plant/bacteria combinations were compared with uninoculated controls. All ineffective combinations grew as badly as the controls.

Table 1. *The strains of* Rhizobium trifolii *studied*

Strain	Type of symbiosis	Source
297/3	Effective	Crimson clover*
297/33	Effective	Crimson clover*
297/32	Ineffective	Crimson clover*
298/533	Effective	Crimson clover*
298/534	Effective	Crimson clover*
298/531	Ineffective	Crimson clover*
298/536	Ineffective	Crimson clover*
C1F	Ineffective	Red clover, Rothamsted, England
161	Ineffective	White clover, Glen Innes, N.S.W., Australia
157	Ineffective	White clover, Glen Innes, N.S.W., Australia

* See Vincent (1954) and Vincent & Waters (1954) for further information about the origin of these strains.

Staining procedures. Preparations of bacteria from nodules were obtained by pressing the surface of cut nodules lightly on to thin coverslips. Fixation, when used, was obtained by floating the coverslip preparations for ½–1 min. on a drop of 2% (w/v) osmic acid in M/15 phosphate buffer (pH 7·0) in a watch-glass. Phase-contrast microscopy of living and fixed material was carried out with a Zeiss Opton system and green light. Nuclear structures were examined by the Feulgen reaction, the HCl + Giemsa method (Robinow, 1942) and by the method of DeLamater (1951). Cell walls were stained with 1% (w/v) crystal violet after 20 min. treatment with 10% (w/v) tannic acid.

Supravital staining reactions were used as follows. Suspensions of the bacteroids were prepared from crushed nodules, the reagents were added and a drop of the mixture mounted for microscopic observation by phase-contrast and bright field illumination. The following cytochemical reagents were used: (1) Janus green B at a final concentration of 0·005% (w/v); (2) Nadi reagent, 1 drop each of 1% (w/v) α-naphthol in 95% (v/v) ethanol and 1% (w/v) aqueous dimethyl-*p*-phenylenediamine added to 1 ml. of a bacteroid suspension; (3) 2, 3, 5-triphenyltetrazolium chloride at a final concentration of 0·05% (w/v); (4) the Gomori method for alkaline phosphatase, adapted as follows. Nodules were crushed in M/15 phosphate buffer (pH 7·0) and the bacteroids centrifuged down. They were then resuspended in a mixture containing 25 ml. 2% (w/v) sodium glycerophosphate, 25 ml. 2% (w/v) sodium veronal, 50 ml. distilled water, 5 ml. 2% (w/v) $Ca(NO_3)_2.4H_2O$ and 2 ml. 2% (w/v) $MgSO_4.7H_2O$. After incubation for 1 hr. at 25° the mixture was centrifuged, the bacteroids rinsed in buffered distilled water (10 ml. M/15 phosphate buffer, pH 7·0; 90 ml. distilled water) and resuspended in 2%(w/v) $CoCl_2.6H_2O$

solution for 5 min. The bacteroids were then washed in two changes of buffered distilled water and resuspended in 0·5 % (w/v) sodium sulphide solution. With this method sites of alkaline phosphatase activity stained brownish black.

Carbohydrate deposits were examined by Lugol's iodine and the Bauer-Feulgen glycogen stain (Glick, 1949).

At all times the morphology and cytology of the bacteroids were compared with the forms seen in microtome sections of the nodules, stained with Heidenhain's haematoxylin.

RESULTS

The structure of effective and ineffective nodules

Stained sections of effective subterranean clover nodules showed an anatomical structure similar to that described for several legume species by Chen & Thornton (1940), Schaede (1932) and others. The meristem of growing nodules was at the outer tip, and next to this were cells which were being penetrated by infection threads. Next were cells in which bacteria, released from infection threads, were undergoing bacteroid formation within the host cell cytoplasm. The bulk of the bacteria-containing tissue of the nodule consisted of vacuolated host cells, whose cytoplasm was tightly packed with mature bacteroids. At the base of the nodule was a region of degeneration which was devoid of bacteroids and which became more extensive as the nodule aged.

The ineffective nodules on subterranean clover were usually smaller than the effective ones, but differed from the description of Chen & Thornton (1940) of such nodules on clover, pea and soya-bean roots, in that the bacterial tissue persisted for some time before the bacteroids disappeared. The period of persistence varied with the strain of ineffective Rhizobium used. The bacteroids in these nodules on subterranean clover were loosely packed in the host cytoplasm and were often irregularly distended. Pl. 1, figs. 1 and 2, show the general arrangement of the tissues in effective and ineffective subterranean clover nodules of similar ages.

Bacteroids from effective nodules

With phase-contrast microscopy considerable intracellular detail is shown. Young bacteroids appeared fairly opaque and to contain several small, dark cytoplasmic granules. In older bacteroids one to four pale central regions were surrounded by darker cytoplasm and several dark granules.

Haematoxylin-stained nodule sections suggested that the bacteroids possess nuclear material, and this was confirmed by the nuclear stains. In the Feulgen preparations a central portion of the bacteroids gave a diffuse positive reaction and contained more densely stained bodies within it. The HCl + Giemsa method showed a variety of basophilic structures. In some preparations there was an unstained central portion of the bacteroids which contained several basophilic bodies. In others the whole of the central portion stained purplish blue. Unhydrolysed bacteroids stained with Giemsa showed a purple central region surrounded by several cytoplasmic granules. DeLamater's method revealed purple-stained chromatin elements within an unstained space surrounded by lightly stained cytoplasm. From this description it seems probable that the central 'perinuclear' region is of nuclear origin. Many different reagents were used in an attempt to determine the nature of the material which made up the perinuclear portion of the bacteroids, but apart from the basophilic properties shown in the unhydrolyzed preparations stained with Giemsa, nothing was found.

By comparing preparations from nodules of various ages, stained by all the methods, the following development of the nuclear structures was deduced. Bacteria released from an infection thread into the host cells contained a pair of nuclear bodies in a rod-shaped cell 2–3 μ. long and *c.* 0·5 μ. wide. Several divisions took place and then the rods began to swell. At this stage the nucleus consisted of a string of chromatinic material, axial to the organisms and divided in branching bacteroids. With continued enlargement the perinuclear regions began to appear and the amount of nuclear material seemed to be decreased as these perinuclear regions enlarged. Just before lysis, which was very rapid, no discrete chromatin was found (Fig. 1). Some bacteroids were seen to contain up to four of these perinuclear regions, each with its chromatinic element or set of elements, but in no case did the cell-wall stain reveal any septation. Stained walls showed variations in thickness which probably arose during the uneven growth which took place during bacteroid formation.

The supravital methods stained the granules which were seen in the cytoplasm of the bacteroids by phase-contrast microscopy. With Janus green B the cytoplasm was stained pale green, and the granules a dense green which passed through the characteristic changes green → pink → colourless which indicate reduction of the dye. The perinuclear region remained unstained by Janus green B. The tetrazolium compound stained the granules a bright red, leaving the rest of the bacteroid unstained. Nadi reagent stained the granules blue at pH 5·7 but only occasionally at higher pH values. The Gomori reaction of the granules was always strongly positive. These granules were very small, and although visible in microscope preparations no satisfactory photographs of them were obtained. From these results it was concluded that the cytoplasmic granules seen in the bacteroids from effective nodules on subterranean clover have most of the properties ascribed to mitochondria-like bodies in other species of bacteria (Mudd *et al.* 1951; Bergersen, 1953) in that they are centres of oxidation-reduction activity, possess cytochrome oxidase as shown by the Nadi reagent, and have a system capable of dephosphorylating glycerophosphate. Similar results were obtained with all the effective strains of bacteria studied, no differences in bacteroid morphology or cytology were seen (Pl. 1, figs. 3–5).

Bacteroids from ineffective nodules

Bacteroids from ineffective nodules differed from effective bacteroids in two major respects: no perinuclear regions developed in the ineffective bacteroids, they showed a considerable accumulation of glycogen. There was a variation in shape as between different strains. Ineffective substrains of nodule bacteria isolated from the subterranean-crimson clover group (Vincent & Waters, 1954), produced bacteroids very similar in shape to the bacteroids produced by the effective parent strains. However, ineffective strains isolated from other *Trifolium* species gave bacteroids in subterranean clover nodules which were less swollen, more variable in shape, and often more branched; this was especially the case with strain C1F. The nuclear material in these ineffective bacteroids was very irregular and usually appeared as a bar of chromatin axial to the cell. With the distortion of the cytoplasm due to accumulation of inclusions, the nuclei became distorted and often were not revealed by the usual nuclear stains. The cytoplasmic mitochondria-like granules of ineffective bacteroids were larger and more prominent than those of effective bacteroids and fewer in number, but they gave the same reactions with the supravital stains.

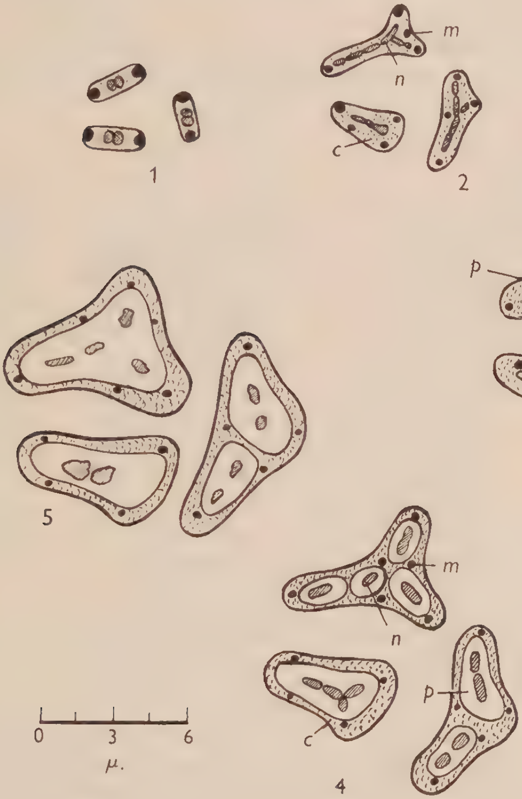

Fig. 1. The development of effective bacteroids: 1, rod-shaped bacteria released from infection threads; 2, beginning of bacteroid formation; 3, beginning of the formation of perinuclear regions; 4, mature bacteroids which pack most of the bacterial tissue of active nodules; 5, old bacteroids before lysis. m = mitochondria-like granules; n = nuclear material; c = cytoplasm; p = perinuclear regions.

The most noticeable feature of the ineffective bacteroids was the accumulation of glycogen. This occurred first on the outside of the mitochondria-like granules, as indicated by a red-brown staining reaction with Lugol's iodine. Inclusions were then formed which usually clustered around the granules as refractile globules in the cytoplasm. These refractile globules enlarged and increased in number as long as the bacteroid itself remained in the host cell; they stained red-brown with Lugol's iodine and gave the characteristic red-violet colour with the Bauer-Feulgen technique. On the contrary, the mitochondria-like granules in effective bacteroids did not stain with Lugol's iodine, and there was no evidence of glycogen accumulation. The relationship between the abundance and degree of persistence of the bacteroids and the extent of glycogen accumulation is shown in Table 2. Most accumulation was found with the strain C1F which persisted as the bacteroid stage in the host cells for more than 50 days. At the end of this time the bacteroids were distended with globules of glycogen (Pl. 1, figs. 7, 10) while with strain 157, which barely persisted for 20 days, accumulation was slight, the only glycogen present being shown as iodine-stained mitochondria-like granules (Pl. 1, fig. 8). The simplified drawings of Fig. 2, made from actual preparations, show some of the types of bacteroids found in ineffective nodules.

Table 2. *The persistence of the bacteroid stage of* Rhizobium trifolii (*ineffective and effective strains*) *in subterranean clover nodules and the presence or absence of glycogen*

Ineffective strains of *Rhizobium trifolii*	Age of nodule (days)			
	20	30	40	50
C 1F	Bacteroids +++ Glycogen +	Bacteroids +++ Glycogen ++	Bacteroids +++ Glycogen +++	Bacteroids +++ Glycogen +++
297/32	Bacteroids ++ Glycogen +	Bacteroids ++ Glycogen ++	Bacteroids and glycogen absent	Bacteroids and glycogen absent
298/536	Bacteroids ++ Glycogen +	Bacteroids + Glycogen +	Bacteroids and glycogen absent	Bacteroids and glycogen absent
161	Bacteroids + Glycogen +	Bacteroids and glycogen absent	Bacteroids and glycogen absent	Bacteroids and glycogen absent
298/531	Bacteroids + Glycogen +	Bacteroids and glycogen absent	Bacteroids and glycogen absent	Bacteroids and glycogen absent
157	Bacteroids ± Glycogen ±	Bacteroids and glycogen absent	Bacteroids and glycogen absent	Bacteroids and glycogen absent
All effective strains	Bacteroids ++++ Glycogen –	Bacteroids ++++ Glycogen –	Bacteroids ++++ Glycogen –	Bacteroids ++++ Glycogen –

Examination of frozen sections of fresh ineffective nodules mounted in Lugol's iodine indicated that there was an inverse relationship between the amount of starch in the host cells and the amount of glycogen accumulated by the bacteroids. Thus strain C1F nodules contained only small amounts of starch but strain 157 nodules contained a great deal which continued to accumulate after the bacteroids had disappeared from the host cell.

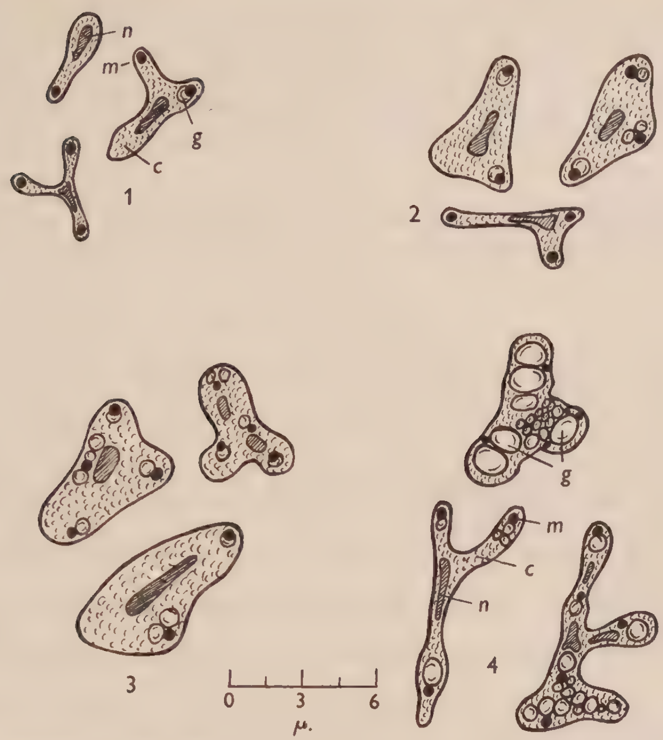

Fig. 2. **Ineffective bacteroids: 1, from strains 161 and 157; 2, from strains 298/531 and 298/536; 3, from strain 297/32; 4, from strain C1F. *g* = glycogen globules, other abbreviations as Fig. 1.**

DISCUSSION

From the foregoing results it may be seen that the distinctive feature of bacteroids from nitrogen-fixing nodules on subterranean clover is the development of the perinuclear regions. Chemical tests did not give any clue as to the constitution of these regions of the organisms, and no theories about their role can be advanced. However, the constant occurrence of these regions in effective bacteroids and their absence in ineffective bacteroids seems significant and may indicate some relation with the nitrogen-fixing processes. The perinuclear regions are linked with the nuclear material which they contain and may be in fact derived from the nucleus. In this connexion it is interesting to observe that the nuclear material possessed no detectable phosphatase activity while the cytoplasmic granules did. This would seem to indicate a low degree of nuclear activity since in most animal and plant cells alkaline phosphatase activity in the nuclei seems to be associated with nuclear activity. This may have a bearing on the non-viability of the bacteroids (Almon, 1933) when transferred to nutrient media.

The general picture of nitrogen-deficient plants is one of carbohydrate excess which is shown by the accumulation of starch in most tissues. It is therefore interesting, but not surprising, that in ineffective nodules in which the bacteroids persist, evidence of carbohydrate excess in the bacteria is shown by the accumulation of the characteristic bacterial reserve carbohydrate, glycogen. Bacteroids which disappear from the host cell at a fairly early stage accumulate little glycogen, but the host cell accumulates a considerable amount of starch.

Thanks are due to Dr P. S. Nutman for advice and criticism, and to Mr J. M. Vincent, University of Sydney, for supplying some of the cultures.

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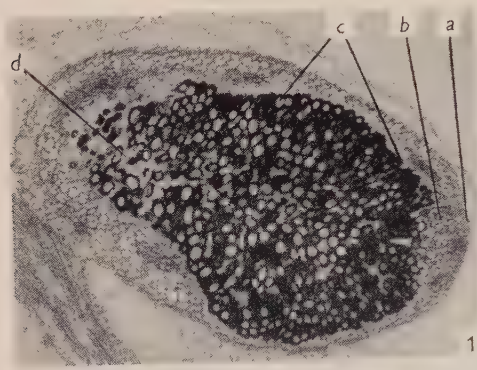

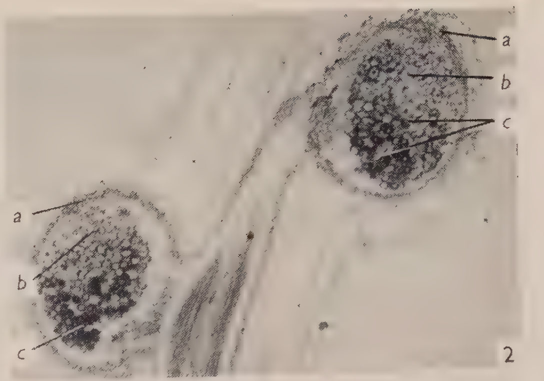

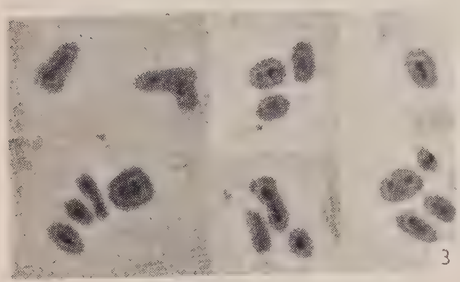

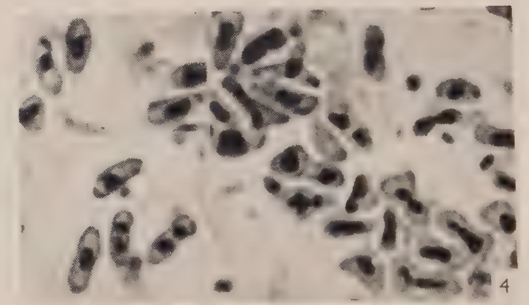

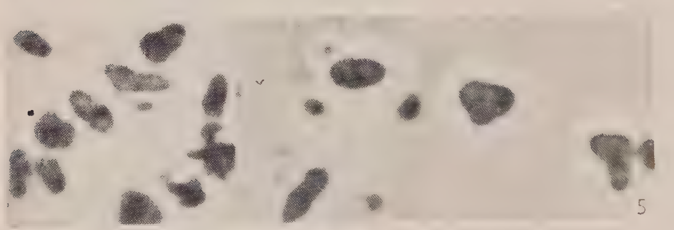

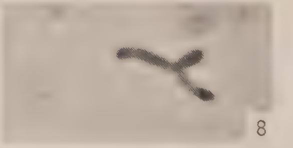

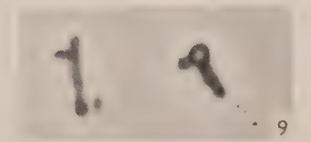

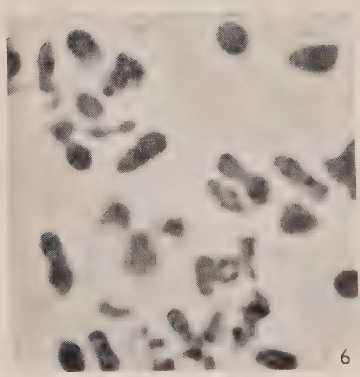

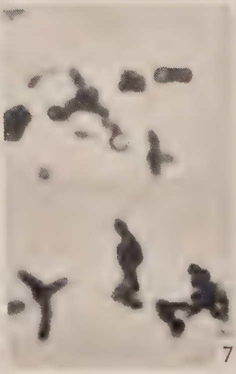

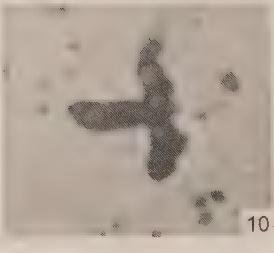

F. J. BERGERSEN—CYTOLOGY OF ROOT NODULE BACTEROIDS. PLATE 1

(*Facing p.* 419)

EXPLANATION OF PLATE

Fig. 1. Twenty-day effective nodule. Haematoxylin. × 160. *a* = meristem; *b* = region of penetration and beginning of bacteroid formation; *c* = region of mature bacteroids; *d* = region of degeneration.

Fig. 2. Twenty-day ineffective nodule showing persistent bacterial tissue (*c*). Haematoxylin. × 160.

Fig. 3. Effective bacteroids; stained by DeLamater's method. × 1600.

Fig. 4. Effective bacteroids; stained HCl + Giemsa. × 1600.

Fig. 5. Effective bacteroids; unfixed and seen by phase-contrast microscopy. × 1600.

Fig. 6. Ineffective bacteroids; strain 297/32; phase contrast. × 1600.

Fig. 7. Ineffective bacteroids; strain C1F; phase contrast. × 1600.

Fig. 8. Ineffective bacteroid; strain 157; phase contrast. × 1600.

Fig. 9. Ineffective bacteroids; strain 161; phase contrast. × 1600.

Fig. 10. Ineffective bacteroid (strain C1F) stained by the Bauer-Feulgen method and counterstained with light green. × 2400.

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Some Factors Affecting the Formation of Iodophilic Polysaccharide in Group D Streptococci from the Rumen

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SUMMARY: Of 25 isolates of α-haemolytic group D streptococci from the rumens of calves (Mann & Oxford, 1955), 7 produced intracellular iodophilic polysaccharide when grown aerobically or anaerobically in agar media containing starches of various origins, amylopectin, maltose, maltotriose, glycogen, amylose and amylopectin dextrins, or amylose glycollate. The iodophilic polysaccharide was not produced from glucose, sucrose, trehalose, glucose-1-phosphate, glucose-6-phosphate, cellobiose or amylose. These results are discussed in the light of the amylolytic action of the bacteria and known enzymic mechanisms of starch-type polysaccharide formation.

In liquid media the polysaccharide was only produced from maltose when the cells were held at a pH value between 5 and 6, either by adjusting the reaction of the medium after the initial growth period, or by transferring the cells to a maltose solution buffered at the correct pH value and incubating further. Under the correct conditions polysaccharide formation was rapid. The polysaccharide was obtained in a partially purified form from the cells and appears to be unlike most 'starches' from bacteria so far reported. A possible explanation of the mode of development of an iodophilic reaction in streptococci in the rumen is given.

The presence in the intestines of animals and birds of bacteria which give a 'starch-' or 'glycogen-' like reaction with iodine in potassium iodide solution has been noted by many workers (e.g. Klein, Schmid & Studt, 1937; Quin, 1943; Baker & Nasr, 1947; Van der Wath, 1948; Baker, Nasr, Morrice & Bruce, 1950; Moir & Masson, 1952; Masson, 1954). Most of these iodophilic organisms are coccoid forms associated with the breakdown of starch granules, and sometimes cellulose, in the digesta, and the iodine-staining polysaccharide of these organisms can easily be seen in wet films. However, a good and rapid production of iodophilic polysaccharide in cultures of intestinal organisms has seldom been attained. MacPherson (1953) isolated 25 strains of amylolytic streptococci from sheep rumen, but these cocci were apparently only feebly- or non-iodophilic *in vitro*. (Later experiments, similar to those described below, showed that they were iodophilic under the correct conditions.) Higginbottom & Wheater (1954) isolated *Streptococcus bovis* from cattle, goat and sheep rumen, and these cocci were stated to produce iodophilic colonies on soluble starch agar after 3–4 days of incubation. Nasr & Baker (1949) showed that washed suspensions of *Clostridium butyricum* from pig caecum gave a strong iodine reaction after incubation for 3 days in buffered glucose-1-phosphate. The synthesis of bacterial polysaccharide in the rumen would appear to be much more rapid than this.

Mann & Oxford (1955) isolated and classified 58 strains of group D streptococci from the rumen of calves of different ages; 42 of these isolates were amylolytic, and of 25 α-haemolytic organisms in this group, 7 produced densely iodophilic colonies when incubated for 3 days on a 0·5 % starch agar medium. These organisms appeared to be identical with those isolated from sheep by MacPherson (1953). Continuing our studies of these and other intestinal bacteria (Hobson & MacPherson, 1952, 1954; Hobson & Nasr, 1951) it was decided to investigate the iodophilic polysaccharide of a typical calf rumen streptococcus (no. 18C6). As a preliminary the substrates for polysaccharide formation were determined. At first it was not possible to obtain densely iodine-staining cells in a liquid medium, so attempts were made to prepare the polysaccharide from cells grown on agar slopes. It was difficult to obtain enough cells in this way, so an investigation was made to determine the conditions necessary for the optimum formation of iodophilic polysaccharide in liquid media. The results of these experiments and a preliminary account of the polysaccharide obtained are given in this paper.

METHODS

The isolation of the organisms used was described by Mann & Oxford (1955). Stock cultures were kept in Robertson's cooked meat broth, and inoculations (4 drops/100 ml.) of the experimental media were made from 24 hr. nutrient broth cultures.

Media. The basal medium consisted of 1 % Bacto yeast extract, 1 % Bacto tryptose, 0·1 % KH_2PO_4, in distilled water, the pH adjusted to 7·6, sterilized by autoclaving at 120° for 15 min.; agar (2 %) was added for plates. Carbohydrates were sterilized by filtration and added in concentrated solution; starches were autoclaved. Where possible the carbohydrates were dissolved in distilled water, but it was found necessary to dissolve some of the starches and dextrins in dilute (*c.* 0·3 N) NaOH in a boiling water bath and then to neutralize the solution with sulphuric acid before sterilizing. In the buffered media the 0·1 % KH_2PO_4 was omitted and appropriate mixtures of KH_2PO_4 and K_2HPO_4 added to give a final concentration of 0·1 M-phosphate. The pH values of the media were checked with BDH Capillators (British Drug Houses Ltd., Poole, Dorset) using at least two indicators where possible.

Carbohydrates. Potato starch was a commercial sample (Hopkin and Williams Ltd., London). For the smooth pea, waxy maize, wheat and maize starches we are indebted to Dr W. J. Whelan, Chemistry Department, University College, Bangor. Holotrich starch (stored by rumen protozoa) was given by Dr A. E. Oxford (Oxford, 1951). The two amylose samples (*a*, *b*, Table 1) were precipitated from potato starch by the thymol-alumina method of Hobson, Pirt, Whelan & Peat (1951). Amylopectin was prepared from potato starch after thymol precipitation of the amylose (Bourne, Donnison, Haworth & Peat, 1948). Soluble starch and glucose were obtained from British Drug Houses Ltd.; maltose hydrate from T. Kerfoot and Sons, Vale of Bardsley; 'dextrine' from G. Gurr Ltd., London; glycogen from L. Light and Co. Ltd., Colnbrook, Bucks. Two samples of dipotassium glucose-1-phosphate were used, one from British Drug Houses Ltd., and the other prepared from potato starch by the method of MacCready & Hassid (1944). Glucose-6-phosphate was used as the barium salt. Maltotriose was prepared by the action of salivary α-amylase on amylose followed by separation of the products on a charcoal-celite column (Whistler & Durso, 1950). Amylose glycollate (1 : 10) was prepared from amylose by treatment in alkali with monochloroacetic acid as described by Peat, Bourne & Thrower (1947) for starch. The dextrins were prepared by acid hydrolysis of amylose or amylopectin until the iodine stain was reddish purple. The solutions were then neutralized, dialysed, the non-diffusible material evaporated to small volume, and the dextrins precipitated by addition of ethanol (5–6 vol.), then washed and dried by trituration with ethanol followed by ether. Portions were used for determination of the blue value (Bourne, Haworth, Macey & Peat, 1948), conversion to glucose by acid, and reducing power as maltose. The absence of oligosaccharides after dialysis and precipitation was confirmed by paper chromatography. The reducing power and iodine stain of the amylose dextrins suggested an average chain length of about 20 units, but the dextrins would be very heterogeneous. The average chain length of the amylopectin dextrin is impossible to assess, because of the branch linkages, but it had a reddish purple iodine stain and a reducing power equivalent to *c.* 4 % of an equal weight of maltose.

RESULTS

Formation of iodophilic polysaccharide on solid media

In the initial experiments all isolates which produced iodophilic colonies on starch were used to test the various carbohydrate substrates. The plates were incubated aerobically for 3 days at 38° as this gave a good iodine reaction in the cells; later it was found that a shorter time of incubation was enough; the results are shown in Table 1. There was good growth on all substrates. A control experiment with 1 % maltose as substrate showed that iodophilic polysaccharide was formed also under strictly anaerobic conditions. The hydrolysis of amylose was complete immediately around the colonies but some material remained unhydrolysed at a greater distance. No polysaccharide was produced from glucose, but it was produced from maltose, and it might be expected from higher analogues of maltose; but an interesting point is that although amylose was hydrolysed by the bacteria, presumably initially to maltose and maltotriose (see Discussion) no iodophilic polysaccharide was formed from it. The only polyglucoses which yielded polysaccharide were all entirely, or preponderantly of a branched chain structure. It seemed worth while to test maltotriose and straight- and branched-chain dextrins and amylose glycollate, the substituent groups of which act like branch linkages to the action of enzymes such as β-amylase. Higher concentrations of amylose and various concentrations of maltose were also included to see whether these affected the production of polysaccharide. Sucrose was tested because a strain of *Clostridium butyricum* produced an iodophilic polysaccharide from this sugar (Hobson & Nasr, 1951). Two typical organisms were used for these tests, and the results are given in Table 2. When no carbohydrate was present in the agar growth was rather scanty and no iodophilic polysaccharide was formed. The growth of the organisms on plates containing glucose-1-phosphate was scanty, so a further test was made to see whether actively growing cells utilized this carbohydrate. Glucose was used for the initial growth to prevent the formation of polysaccharide which might have rendered later results difficult to interpret. Glucose (2%) plates were inoculated and incubated; at 24 hr. intervals a plate was taken and a sterile filter-paper disk dropped on to a portion of the agar having a considerable number of colonies. The paper was then saturated with sterile 5% glucose-1-phosphate solution and the plate incubated for a further 24 hr. to allow the glucose-1-phosphate to diffuse into the surrounding medium. It was expected that if glucose-1-phosphate were utilized at all a suitable concentration would be found at a certain distance

Table 1. *Formation of intracellular iodophilic polysaccharide by rumen streptococci on solid media*

Basal medium given in text. All carbohydrates in 0·5% concentration. Plates incubated 3 days aerobically at 38°. C = complete hydrolysis of substrate (i.e. lack of colour on flooding plate with I_2 + KI solution; P = partial hydrolysis; +, ++, etc. = degrees of iodine colour of colonies; +++ = blue black; – = no colour; * = not tested.

	Organisms						
Substrate	18C6	19C1	16M1	18C2	18M1	18C7	18M2
	Reactions						
	C	C	P	C	C	C	C
'Dextrine'	+	+	+	+	++	++	++
Glucose	–	–	–	–	–	–	–
Glycogen	C	C	C	C	C	C	C
	++	+	+	++	+	+	++
Maltose	++	+	+	+++	+++	+++	++
	C	C	C	C	C	C	C
Holotrich starch	+	+	+	+	±	++	++
	P	P	P	P	P	P	P
Amylose, *a*	–	–	–	–	–	–	–
	P	P	P	P	P	P	P
Amylose, *b*	–	–	–	–	–	–	–
	C	C	C	C	C	*	C
Smooth-pea starch	+	+	+	+	+		+
	C	C	C	C	C	C	C
Waxy-maize starch	+	+	+	+	+	+	++
	C	C	C	C	C	C	C
Soluble starch	++	++	++	++	++	++	++
	C	C	C	C	C	C	C
Wheat starch	++	++	++	++	±	±	+
	C	C	C	C	C	C	C
Maize starch	++	++	++	++	++	+	++
	C	C	C	C	C	C	C
Amylopectin	+	+	+	++	++	+	++
	C	C	C	C	C	C	C
Potato starch	+	+	+	++	+	++	+

from the paper and the colonies in that region would become iodophilic. This experiment was repeated on plates incubated for 24, 48, 72 and 96 hr. after the initial inoculation, but no iodophilic polysaccharide was formed. This suggests that the cells grown on glucose were unable to utilize glucose-1-phosphate.

Table 2. *Formation of intracellular iodophilic polysaccharide by rumen streptococci on solid media*

Basal medium given in text. Plates were incubated for 3 days aerobically at 38°.

Substrate (%, w/v)	Organism	
	18C6	19C1
	Reactions	
Cellobiose (0·5)	–	–
Maltose (1·0)	+++	+
Maltose (0·1)	–	–
Glucose (1·0)	–	–
	C	C
Amylose dextrin (*a*; 0·5)	–	–
	C	C
Amylose dextrin (*b*; 1·0)	+++	+
	C	C
Amylopectin dextrin (1·0)	+++	+
Maltotriose (2·0)	+++	*
	P	
Amylose (1·0, 2·0)	–	*
	C	
Amylose glycollate (1:10; 0·5)	+++	*
Sucrose (2·0)	–	–
Trehalose (0·5)	–	–
Glucose-1-phosphate (0·5, 2·0)	–†	–†
Glucose-6-phosphate (0·5)	–†	–†

† = growth scanty; other symbols as in Table 1.

After 17½ hr. incubation on 2% maltose agar a good growth was apparent, but the colonies were not iodophilic; after a further 6 hr. the colonies were strongly iodophilic, and the iodine stain increased in intensity on further incubation. After 4 days the colonies seemed to contain many dead and disintegrating cells, and observations were not continued. In some starch and glycogen plates conditions appeared to become suitable for iodophilic polysaccharide formation after about 2 days. The appearance of iodophilic and non-iodophilic colonies is shown on Pl. 1, fig. 1.

Formation of iodophilic polysaccharide in liquid media

In a preliminary attempt to prepare the polysaccharide, organisms 18C6 and 19C1 were each inoculated into two basal broths containing respectively 2% maltose and 2% glucose, and incubated aerobically for 7 days. Tests at daily intervals showed that the organisms from the glucose medium contained no iodophilic polysaccharide, and those from the maltose medium only a small amount which did not increase with time of incubation. It was evident, therefore, that the conditions in the liquid medium were not as favourable to the formation of polysaccharide as on the solid medium. Since the constituents of the media were the same, except for the agar, it did not seem likely that a nutritional factor was lacking in the liquid medium, and of other possibilities the pH value of the medium was the main variable. The initial pH value of the maltose broths was 7·6, but this rapidly decreased to 4·4 during the growth of the cells. As a first experiment, therefore, an attempt was made to keep the medium only slightly acid. Two broths containing 2% maltose, one buffered at pH 7 and the other unbuffered (pH 7·6) were inoculated and incubated in screw-top bottles. (In the following experiments the media were incubated in sealed bottles, without special precautions to exclude air on opening and closing the bottles; these are referred to as aerobic incubations.) The results suggested that more polysaccharide might be formed if the medium were kept at a pH value of 5–5·5 after the initial growth period.

Formation of polysaccharide by cells in buffered maltose broths

Basal broth containing 2% maltose and buffered at different pH values was inoculated with streptococcus 18C6. At intervals, portions of the cultures were removed and centrifuged to test the iodine stain of the cell sediments, the microscopic appearance of the cells, and the pH value and reaction with Fehling's solution of the supernatant liquids. The results are shown in Table 3. As

Table 3. *Formation of intracellular iodophilic polysaccharide by a rumen streptococcus in buffered liquid media*

The medium is the basal one given in the text with the addition of maltose (2%, w/v). Iodine stains: g = green; pb = purple black; y = yellow; bl = greyish blue.

The cells were capsulated at all times and Fehling's test showed some sugar present in the cultures after 42 hr.

Time of incubation (t) (hr.)	pH value		Iodine stain of bacterial sediment
	Initial	At time t	
17	5·5	4·5	g
	6·0	4·3	pb
	6·3	5·0	g
	7·1	6·6	y
	7·6*	4·1	g
23	5·5	4·4	g
	6·0	4·2	pb
	6·3	4·4	bl
	7·1	6·6	y
	7·6	4·2	g
42	5·5	4·4	yg
	6·0	4·3	pb
	6·3	4·4	yg
	7·1	4·3	bl
	7·6	4·4	y

* Unbuffered medium as control.

cells fill up with polysaccharide the iodine colour of the sediment changes from yellow to green, blue and almost black. The pH value of the unbuffered medium fell rapidly to 4·2 and little iodophilic polysaccharide was produced. In the media initially buffered at pH 6·3 and 7·1 the polysaccharide content increased when the pH was about 5–6. After the pH value had fallen to 4·4 the organisms rapidly died, and after about 24 hr. at pH 4·4 the cultures were sterile. In some cases the iodine colour seemed to fade when the cells were kept at an acid pH value, probably due to autolysis of the cells and digestion of the polysaccharide by cell enzymes. In a liquid culture initially buffered at pH 6 and incubated strictly anaerobically, the formation of polysaccharide was similar to the corresponding aerobic cultures, but the growth was not quite as dense and the pH value of the medium did not fall below 4·8.

Formation of polysaccharide by cells suspended in buffered maltose solutions

Organism 18C6 was grown aerobically in 2 % maltose broth buffered at pH 6, for 17 hr., divided into portions (20 ml.), centrifuged, and each sediment of organisms resuspended in 2 % maltose solution buffered with 0·1 M-phosphate (20 ml.) to the pH values given in Table 4. The suspensions were incubated for a further 22 hr. and then centrifuged, and the supernatant liquids and cell sediments examined. In each broth culture the pH value of the medium fell to 4·2–4·4 during incubation, the iodine colour of the cell sediment was green, and sugar was still present in the medium. The results of the further incubations are shown in Table 4. The pH values of the buffered maltose solutions did not change during the incubation. At pH values of 6·1, 5·6 and 5·1 the intensity of the iodine stain of the cells increased markedly on incubation, with 17, 28 and 17 % respectively of the total cells becoming iodophilic. In similar experiments, where the organisms were transferred to 2 % glucose-1-phosphate at pH 5·5 and buffer (pH 5·6) alone, no iodophilic polysaccharide was formed.

Table 4. *Formation of iodophilic polysaccharide by a rumen streptococcus suspended in buffered maltose solutions after growing* 17 *hr. in a* 2 % *maltose broth*

Symbols for iodine stains as in Table 3. Initial iodine stain green, i.e. the cells contained a little iodophilic polysaccharide.

pH value of maltose solution	Iodine stain of cell sediment*
6·8	yg
6·1	pb
5·6	pb
5·1	pb
4·5	g
3·7†	yg‡

* After incubation for 22 hr. in buffered maltose solution.
† pH brought down by addition of HCl.
‡ Cells coagulated on bottom of bottle.

Since older organisms formed polysaccharide under the correct conditions it appeared of interest to see whether young rapidly growing cells formed polysaccharide when exposed to a lowered pH value in buffered maltose solutions. The experiment was repeated, and the initial maltose broth was incubated for only 5 hr. At this time, although the growth of organisms was good, the pH value of the medium had fallen only to 5·8 and there was no detectable iodine stain in the cells. The results of incubating organisms in buffered maltose for a further 16 hr. are shown in Table 5. (Similar results were obtained after incubation for 22 hr.). The pH values of the maltose solutions did not alter during the incubation. Again incubation at pH values of 5–6 favoured the formation of iodophilic polysaccharide.

Table 5. *Formation of iodophilic polysaccharide by rumen streptococci suspended in buffered maltose solutions after growing 5 hr. in a 2 % maltose broth*

Symbols for iodine stains as in Table 3. Initial iodine stain yellow (y), i.e. the cells contained no iodophilic polysaccharide.

pH value of maltose solution	Iodine stain of cell sediment*
6·8	y
6·1	y
5·6	pb
5·2	pb

* After incubation for 16 hr. in buffered maltose solution.

Table 6. *Total and viable counts of rumen streptococci forming polysaccharide in buffered maltose solutions*

The organisms were grown for 17 hr. in a 2 % maltose broth, centrifuged off, resuspended in buffered maltose solutions (2 %, w/v) and incubated further. Counts were made by the methods described in the text.

Buffered maltose solution (pH value)	Time of incubation (hr.)										
	0	4	6	10	24	48	0	6	24	0	24
	Iodophilic cells (%)						Viable cells/ml.			Total cells/ml. $(\times 10^{-6})$	
5·2	<1	—	—	—	21	—	—	—	—	—	—
5·7*	<1	13	17	19	16	—	500	0	0	960	1120
5·7	<1	13	18	18	15	—	4900	0	0	960	1040
5·7	<1	—	—	—	12	10	—	—	0	—	—

* Cells washed before resuspending in buffer. — = not tested.

In a repeat of the experiment where portions of a 17 hr. culture were resuspended in buffered maltose solutions, viable and total counts and counts of the number of iodophilic organisms were made. The organisms in one portion of the culture were washed twice in sterile saline before resuspension and one suspension was incubated for 48 hr. The results (Table 6) are similar to others obtained, but the percentage of iodophilic cells was rather lower than was often found. Viable counts were made by the pour-plate method in 0·5 % maltose agar. Total counts were made on culture dilutions stained with methylene blue on a haemocytometer slide. Iodophilic bacteria were counted on 500 cells in different parts of iodine-stained wet films. The results showed that the cells in a medium favourable for polysaccharide formation were nearly all non-viable in a medium which usually supported their growth.

Formation of polysaccharide in cultures after daily adjustment of pH *value*

This experiment was repeated a number of times, and although the percentage of iodophilic organisms varied from about 20 to 35 % at 42 hr. the general trend was always the same. A typical experiment is described below. Organism 18C6 was inoculated into 100 ml. of a medium containing 2 % maltose, buffered at pH 6, and the culture incubated aerobically. At 18 hr. the pH value of the medium had fallen to 4·0 and the iodine colour of the sediment was green, with 1 % of iodophilic cells. The pH value was adjusted to 5·7 with NaOH and incubation continued. At 24 hr. the pH was 5·4 and the iodine colour of the cell sediment purple. At 42 hr. the pH had fallen to 4·2 and the iodine colour of the cell sediment was a dark purple with 33 % iodophilic cells (Pl. 1, fig. 2). The pH value was again adjusted to 5·7 and incubation continued, at 48 hr. the pH being 5·6. At 66 hr. the pH was 4·4 and the number of iodophilic cells 49 % of the total. The pH value was readjusted, but by this time there was only a trace of sugar in the medium, and at 90 hr. the pH value had fallen only to 4·8. The number of iodophilic cells had also decreased to 38 % and many cells were disintegrating.

It was noted that when the cultures had become suitable for polysaccharide formation the cells had become Gram-variable and many were irregular in appearance like cells suspended in buffered maltose. Thus it seemed of interest to determine the number of viable bacteria in these cultures forming polysaccharide. A 2 % maltose broth buffered at pH 6 (100 ml.) was inoculated as above and incubated for 17 hr.; the pH value of the medium had then fallen to 4·2 and the iodine stain of the cell sediment was green. The pH was raised to 5·7 and the culture reincubated. At the times stated in Table 7 viable and total counts were made. After incubation for 24 hr. the pH had fallen to 4·5, and the iodine stain of the cell sediment was a deep purple with 33 % of the cells iodophilic. In another similar experiment the total number of viable organisms initially present was much higher, but there was a more marked fall in numbers on adjusting the pH value. This was followed by a rapid increase in numbers at about 5–6 hr. as in Table 7, and the total and viable counts after 24 hr. were almost the same as those given in the table.

In similar experiments, where the pH value of a 2 % glucose broth was raised to 5·7 every 24 hr., no iodophilic polysaccharide was produced. An attempt was also made to prepare a 2 % amylose broth by dissolving amylose in water by autoclaving and adding the remainder of the constituents as a concentrated solution, but the amylose began to retrograde almost immediately, and although a heavy inoculum was made into the medium no growth occurred. The experiments were therefore abandoned.

Partial purification of the iodophilic polysaccharide

In initial experiments the isolation of the polysaccharide was attempted by autoclaving the whole cells in neutral solution, and by extracting with sodium hydroxide at different concentrations and temperatures. Very little of the polysaccharide was extracted by these methods so it was decided to try to rupture the cells and then extract the polysaccharide with chloral hydrate. Shaking in suspension in a tissue disintegrator (Mickle, 1948) with ballotini no. 12 glass beads (Chance Ltd., Smethwick), or grinding a dry powder of bacteria *in vacuo* with glass beads (Gunsalus & Umbreit, 1945) followed by extraction, gave very small yields of polysaccharide. A method giving more disintegration of the cells and a better yield of polysaccharide was afforded by grinding the wet cells with alumina (McIlwain, 1948), followed by extraction. However, it was found that the alumina dissolved in the chloral hydrate used for extraction, and was later reprecipitated, giving polysaccharides with a high ash content. The alumina was therefore replaced by carborundum powder. A partial purification of the polysaccharide has been achieved by this method which is described briefly below.

Table 7. *Viable and total counts during the formation of iodophilic polysaccharide in rumen streptococci in a liquid medium after adjustment of the* pH *to* 5·7

The organisms were grown for 17 hr. in a 2% maltose broth, the pH of the broth was then raised to 5·7 and incubation continued. Counts were made by the methods described in the text.

Time (hr.)	Viable cells /ml. $\times 10^{-3}$	Total cells /ml. $\times 10^{-6}$
0*	25	800
1†	16	720
2	20	960
3	35	960
4	55	880
5	75	800
6	270	880
7	1500	960
24	5510	1520

* Before raising pH. † Immediately after raising pH to 5·7.

The streptococcus 18C6 was grown aerobically for 4 days on agar plates of basal medium + 2% maltose. The colonies were then washed off the plates with a jet of water, filtered through glass wool and centrifuged. The organisms were resuspended in water, again filtered, and centrifuged. After washing twice more with water the organisms were frozen to −20° overnight and the solid mass mixed with twice its weight of cold carborundum (Grade 4F; Griffin and Tatlock Ltd., London) in a mortar cooled to 0°, and then ground by hand for 15 min. A little more carborundum was added when necessary to keep the paste almost solid, and the mortar cooled at intervals. The whole mass was next extracted twice with 33% (w/v) chloral hydrate at 80° (Meyer & Bernfeld, 1940). The chloral hydrate extract, after centrifuging, was filtered through a sintered glass filter (grade 5), then poured slowly into well-stirred excess acetone (3–4 vol.), and the whole allowed to stand overnight at 2°. The precipitated polysaccharide was removed by centrifuging, washed and dried by trituration with acetone and ether. Such preparations contained about 0·8 % nitrogen and 13 % ash. To decrease the protein and ash contents the following procedure was carried out.

Several combined preparations were wetted with ethanol and dissolved in 0·1 N-NaOH by heating in a boiling water-bath for a few minutes. After neutralizing, to each 25 ml. was added hydrated sodium acetate (1 g.), butanol (0·5 ml.), and chloroform (5 ml.). The whole was shaken for 10 min. and centrifuged. The aqueous layer was dialysed for 2 days against running distilled water; then the non-diffusing polysaccharide was precipitated with ethanol (2 vol.), spun down, and dried by trituration with ethanol and ether.

The yield in the first stage of the preparation was very small, only some 5 % of the dry weight of bacteria. A typical analysis of the polysaccharide was: 'starch' content, 59·2 %, from reducing sugar estimated as glucose by a copper reagent (Somogyi, 1952) in conjunction with Nelson's reagent (1944), after acid hydrolysis under the conditions described for potato starch by Pirt & Whelan (1951); blue value, 17·1 'Eel' colorimeter units (Hobson & MacPherson, 1952); total nitrogen (micro-Kjeldahl), 0·2 %; ash after total combustion, 11·1 %. The main sugar in the hydrolysate was glucose, but some preparations contained a trace of galactose and possibly rhamnose. By the formamide chromatogram technique of Nussenbaum (1951) the polysaccharide was shown to contain some short-chain iodine-staining dextrins which migrated with the solvent front. The conversion to reducing sugars, estimated as maltose, by soya-bean β-amylase was 68 % of the estimated 'starch' content, suggesting the presence of some amylopectin type polysaccharide. A small amount of maltotriose was detected in the products of β-amylolysis which might have come from dextrins of an uneven number of glucose units. The properties of the different impure preparations of the polysaccharide so far made seem to be similar, but all the polysaccharide is not extracted from the cell sediment. The nature of the unhydrolyzable material in the polysaccharide preparations is at present unknown, but it is hoped that this can be removed when the polysaccharide is prepared on a larger scale from liquid cultures.

DISCUSSION

The formation of intracellular iodine-staining polysaccharide in some calf rumen streptococci has been demonstrated. However, all the organisms were capsulated before visible formation of iodophilic polysaccharide took place (and also in glucose broths) and the capsules appeared to persist even when many iodophilic bacteria were present. This capsule is most probably mainly polysaccharide in nature (Hobson & MacPherson, 1954). It is also possible that other polysaccharides are present in the cells, but in the following discussion 'polysaccharide' signifies intracellular iodine-staining material.

In an investigation of the amylase of a sheep rumen streptococcus, which appears to be identical with the calf rumen organisms used here, it was shown that the principal products of the hydrolysis of the amylose component of starch were maltose and maltotriose (Hobson & MacPherson, 1952); the same products, and some branched-chain oligosaccharides, are formed from amylopectin. As expected, since the rumen streptococci build up iodine-staining polysaccharide when digesting starches *in vivo* and *in vitro*, the maltose and maltotriose formed during the hydrolysis of starch can be utilized for the synthesis of polysaccharide. Glucose, which is formed in only small amount by the rumen streptococcus amylase, is not so utilized. The maltotriose preparation contained traces of maltose and glucose, but the amount of maltose present would not alone be sufficient to form polysaccharide. There are two possible methods of enzymic formation of 'starch' from maltose, one by an amylomaltase type of enzyme (Monod & Torriani, 1948) which produces polyglucose and glucose from maltose; the other by a maltose phosphorylase producing α-glucose-1-phosphate from maltose, and a phosphorylase converting the glucose-1-phosphate to amylose. Fitting & Doudoroff (1952) made enzyme preparations from *Neisseria meningitidis* which produced β-glucose-1-phosphate from maltose. Both enzyme systems would then need a type of Q-enzyme to form the amylopectin component of a normal starch. The second possibility appears to be ruled out by the lack of utilization of glucose-1-phosphate by cells grown on glucose or maltose. Amylomaltase forms mainly reducing oligosaccharides 4–6 glucose units in length, unless the glucose formed at the same time is removed by the glucose oxidase notatin, when a blue iodine-staining polysaccharide is formed (Monod & Torriani, 1948; Doudoroff, Hassid, Putman, Potter & Lederberg, 1949). If an amylomaltase type of enzyme operates in the streptococci then the glucose formed must be utilized by the cells, as only maltotriose and a little material of smaller R_F value was detected in chromatograms of an extract of the agar or of the water used to wash the colonies from the agar, during the preparation of the polysaccharide. Doudoroff *et al.* (1949) found no glucose in the medium when *Escherichia coli*, containing an amylomaltase, utilized maltose. It seems likely that maltotriose is an intermediate in the formation of bacterial polysaccharide from maltose, and this suggests that only glucose units are added to the growing molecules during the formation of polysaccharide, and not maltose units as such.

It might be expected that all dextrins and starch fractions would be equally effective in the formation of polysaccharide by these organisms, but apparently the linear molecules are not easily utilized, even though they support the growth of the bacteria. The simplest explanation of this is the poor solubility of the amylose type molecules. In a liquid medium amylose rapidly retrogrades and in the agar media, although there was sufficient amylose in solution to give a dense iodine colour before hydrolysis by the growing bacteria, some of it was out of solution, and it is quite possible that after the hydrolysis products had been utilized for growth of the bacteria there was not sufficient sugar left for the formation of polysaccharide (a certain minimum concentration of maltose is needed; Table 2). Increasing the concentration of amylose in the plates might not proportionately increase the amount of amylose actually in true solution and available for the bacteria. Increasing the concentration of amylose dextrins, the smaller molecules of which do not retrograde as easily as the large ones, might greatly increase the amount of polysaccharide in solution. Amylose glycollate is easily soluble and does not retrograde, whilst amylopectin, glycogen and native starches are all more stable in solution than separated and alcohol dried amylose. It does not appear that an unfavourable pH value alone was responsible for the non-production of polysaccharide because flooding some of the colonies on a 2% amylose plate, after 2½ days of growth, with buffer at pH 5·7, did not induce the formation of polysaccharide.

From the tests of liquid media it would seem that the pH value of the colonies on agar plates must become stabilized at 5–6 to allow the formation of polysaccharide. An attempt was made to test this point, but all that could be said was that the general pH of the plates was about 6; the pH value of the colonies was not ascertainable.

When the plate cultures were flooded with iodine it was noted that in many cases only the centre of the colonies stained blue, the outer zones of, presumably, younger cells were almost colourless. The results of viable counts showed that after some hours at a low pH value the cells lost their ability to reproduce in a medium in which they usually grew rapidly. In young cultures in liquid and solid media no iodine-staining cells were seen. These and other results suggest that the ability to store polysaccharide is possessed only by non-viable or resting organisms, and is not connected with a rapid growth of the bacteria. These conclusions are supported by the work of Smith & Baker (1944), who found that the number of iodophilic organisms which accumulated during the incubation of rumen contents with maltose *in vitro* was more than might be assumed to result from multiplication alone. It appeared, therefore, that the storage of polysaccharide was taking place not, or not only, in dividing organisms but in many of those already present. It is possible that polysaccharide is being produced in all organisms in maltose or starch media, but that it is rapidly utilized by growing organisms, and an acid medium, or one containing no nitrogen source for growth, causes the inactivation first of enzymes of utilization and polysaccharide accumulates until the enzymes forming it become inactive.

The general pH value of the sheep's rumen is about 6–7. This appears to be unfavourable for the formation of bacterial polysaccharide by the streptococci. However, the bacteria attacking starch and cellulose are found in close contact with these materials, very often in cavities (see photomicrographs in Klein *et al.* 1937; Baker & Nasr, 1947; Baker *et al.* 1950). The rapid formation of acids by the organisms might cause the immediate surroundings of the bacteria to be at a lower pH value than the general body of the rumen and thus the conditions for polysaccharide formation might be present.

The formation of iodophilic polysaccharide from maltose appears to be a property of other amylolytic group D streptococci. The sheep rumen streptococci formed polysaccharide under the correct conditions as did *Streptococcus bovis* no. 8177 and 8133 (National Collection of Type Cultures). *S. faecalis*

(NCTC 775) and *S. faecalis* isolated from the rumen of the sheep in our own laboratories were non-iodophilic under the same conditions, but they were non-amylolytic.

The iodine-staining polysaccharides formed by bacteria vary in composition. Aubert (1951) isolated a glycogen-like polysaccharide from *Bacillus megaterium*. The starch of *Corynebacterium diphtheriae*, like normal plant starch, is composed of amylose and amylopectin (Carlson & Hehre, 1949), and the starch of a rumen strain of *Clostridium butyricum* seemed to be amylopectin-like in character (Hobson & Nasr, 1951). The composition of the iodophilic polysaccharide of these rumen streptococci is not completely elucidated, but the results above show that it is 'starch-like' in that it is a polyglucose with presumably α-1 : 4 linkages as it gives an iodine stain and is susceptible to hydrolysis by β-amylase. The incomplete hydrolysis by β-amylase suggests that some branch linkages are present, but the extent of the hydrolysis and also the iodine colour and blue value (*c.* 17) precludes the presence of only amylopectin. The blue value of amylopectin on the Eel colorimeter scale is about 5, that of potato starch about 16, and amylose about 50. Whilst the blue value of the polysaccharide is about that of potato starch the colour of the iodine stained solution at this concentration is purplish, unlike that of starch, and this and the results of the formamide chromatograms suggest the presence of molecules of short chain length. It is possible that these represent stages in the formation of the polysaccharide and that their amount may vary in different cells. From the results obtained to date it would appear that this bacterial polysaccharide does not fit into any of the usual classifications of starch, amylose and amylopectin or glycogen, and its properties will be the subject of further investigations.

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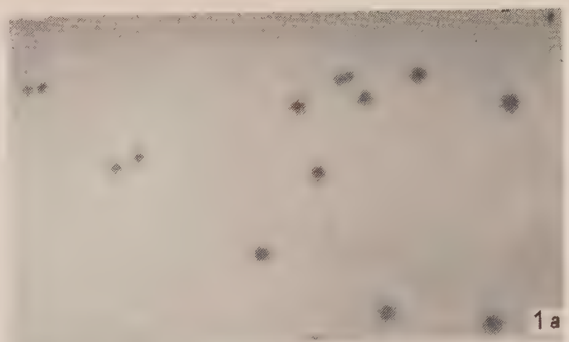

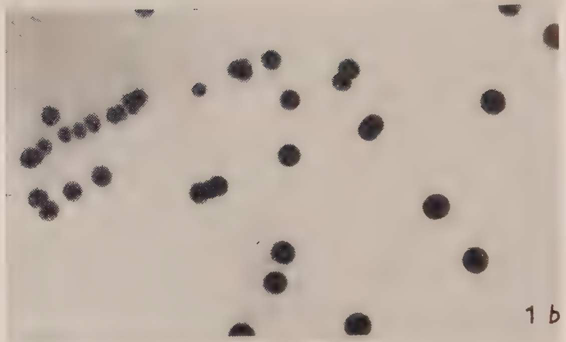

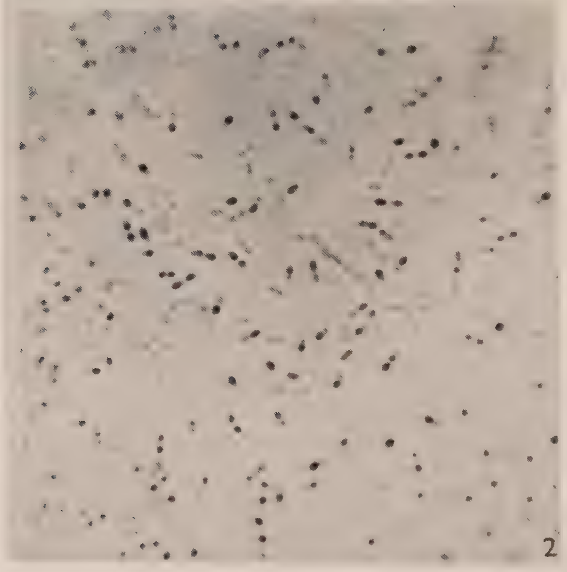

P. N. HOBSON & S. O. MANN—IODOPHILIC STREPTOCOCCI. PLATE 1

(Facing p. 435)

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EXPLANATION OF PLATE

Fig. 1. Iodine-stained colonies of rumen streptococci after 3 days on (*a*) glucose, (*b*) maltose agar. Colonies on maltose with iodine staining cells are blue-black, those on glucose are yellow-brown. × 2.

Fig. 2. Wet preparation, iodine stained, of rumen streptococci from maltose broth 24 hr. after adjustment of pH from 4·0 to 5·7. Iodophilic polysaccharide appears black. × 1000.

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Factors Influencing the Antibacterial Action of Spermine and Spermidine on *Staphylococcus aureus*

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SUMMARY: Spermine and spermidine inhibited the growth of *Staphylococcus aureus* under both aerobic and anaerobic conditions. The activity of spermine was decreased by sera of different species; sheep and bovine serum showed most effect. This antagonistic effect was destroyed by heating the sera at 100° for 30 min. Sodium and potassium also decreased the antibacterial effect of spermine and spermidine. Spermine exerted a bactericidal effect on staphylococci in broth at 37° and at 6°, but not on washed organisms suspended in buffer at 6°. The dependence of the bactericidal effect of the bases seems to depend on some metabolic activity of the cocci.

The antibacterial action of spermine and spermidine was described previously (Gurevitch, Rozansky, Weber, Brzezinsky & Eckerling, 1951; Rozansky, Bachrach & Grossowicz, 1954; Rozansky, Razin & Grossowicz, 1954); Hirsch & Dubos (1952) studied the antimycobacterial effect of these substances. According to Hirsch (1953*a*, *b*) spermine itself was not antimycobacterial but a derivative of it, obtained by the action of an oxidase present in sheep and bovine sera, was the active agent. In the present paper evidence is presented that spermine itself is bactericidal against staphylococci.

METHODS

The technique described previously was used (Rozansky, Bachrach & Grossowicz, 1954). Spermine tetrahydrochloride and spermidine phosphate (Hoffman-La Roche Inc.) were used. The antistaphylococcal tests were made in Difco nutrient broth. The pH value of the medium was adjusted by adding sodium hydroxide or hydrochloric acid and was measured by a Beckman pH meter. Nutrient broth containing increasing twofold dilutions of spermine or spermidine was distributed into 2 ml. quantities in 100 × 12 mm. test tubes. The tubes were plugged with cotton-wool and autoclaved for 20 min. at 15 lb./sq.in.

Staphylococcus aureus strain 23 was the main test organism; 0·1 ml. of a $1/10^4$ dilution of a 24 hr. broth culture was used as inoculum. The tubes were incubated at 37°, unless otherwise specified. The antibacterial activity was expressed as the minimal concentration of spermine or spermidine which caused complete inhibition of growth visible to the naked eye. In experiments to test the bactericidal effect of spermine, a plate count technique was used.

Anaerobic tests were performed in a jar from which the air was evacuated and the remaining oxygen removed by pyrogallol and NaOH; reduced methylene blue was used as indicator of anaerobiosis.

RESULTS

Effect of spermine and spermidine under anaerobic conditions

To test whether the action of spermine and spermidine was affected by oxidative enzymes of the cocci or by the presence of oxygen in the medium, experiments under anaerobic conditions were performed. As *Staphylococcus aureus* strain 23 grew very poorly under anaerobic conditions, *S. aureus*, Oxford strain, was used and to obtain better growth, the broth (pH 7·4) was enriched with glucose to 0·5 % (w/v). A similar experiment under aerobic conditions was run simultaneously.

Spermine and spermidine both showed the same degree of antibacterial activity under aerobic or anaerobic conditions. After 24 hr. incubation at 37°, $1{\cdot}8 \times 10^{-4}$ M-spermine (60 μg. spermine tetrahydrochloride/ml.) and $1{\cdot}8 \times 10^{-3}$ M-spermidine (1000 μg. spermidine phosphate/ml.) completely inhibited the growth of *Staphylococcus aureus*, Oxford strain. These results were confirmed in two other similar experiments, and with a different strain of *S. aureus*.

Effect of various unheated and heated sera

To evaluate any potential antibacterial role of spermine *in vivo*, it was desirable to investigate the influence of serum on the system. Since serum contains both heat labile components and heat stable components, the action of unheated and of heated sera was examined.

To test the effect of serum on the antistaphylococcal activity of spermine the following procedure was adopted: sera were diluted 1/4 with distilled water and sterilized by Seitz filtration. A parallel series of dilutions of serum was heated in boiling water for 30 min. Serial dilutions of spermine were made in broth containing the equivalent of 5 % (v/v) of unheated or of heated serum. The pH value of the final mixtures was adjusted to 7·8. The tubes were inoculated and the results read after incubation at 37° for 48 hr.

The effect of the various sera is indicated in Table 1. All the unheated sera at 5 % (v/v) antagonized the action of spermine; sheep and bovine sera decreased the activity 16- and 8-fold, respectively; guinea-pig and rabbit sera were much less active; the sample of human serum showed an intermediate effect. On the other hand, heated sera (human, sheep, guinea-pig, rabbit) did not affect the action of spermine; only the heated bovine serum retained a little antagonistic activity.

To simulate conditions in the animal body more closely, the effect of undiluted serum was tested. Samples of sera of the same species were pooled, sterilized by Seitz filtration and put into tubes, the first receiving 1·8 ml., and the following tubes 1 ml. of serum. Spermine tetrahydrochloride was added to the first tube to give a final concentration of 2 mg./ml.; decreasing concentrations of spermine were then obtained in successive tubes by transferring 1 ml. amounts from one tube to the next. All tubes were inoculated, and the results are read after 48 hr. of incubation at 37°. The results are shown in Table 1. Undiluted serum of man, guinea-pig and rabbit decreased the antibacterial activity of spermine against *Staphylococcus aureus* slightly, as compared with sheep and bovine serum; 60–125 μg. spermine.4HCl/ml. being inhibitory in the sera of man, guinea-pig and rabbit, while *c.* 10 times this amount of spermine was necessary to inhibit growth in sheep and bovine sera.

Table 1. *The effect of heated and unheated sera of various species in annulling the toxicity of spermine for* Staphylococcus aureus

Inoculum used: *c.* 10^4 organisms/ml. Heated serum was serum diluted 1/4 with distilled water and heated for 30 min. in boiling water. All media were adjusted to pH 7·8. Final concentration of sera 5 % (v/v).

The results were read after 48 hr. incubation at 37°, a positive inhibitory effect on growth meaning no growth of the test organism visible to the naked eye. The coefficient of inhibition of spermine activity by added serum (last column) is the degree of decrease in spermine activity as compared with the control without added serum (=1); 16 = 16-fold decrease, etc.

Sera added to medium	Minimal concentration of spermine.4HCl which inhibited growth in presence of serum (μg./ml.)	Coefficient of inhibition of spermine activity by serum
Unheated		
Sheep	500	16
Bovine	250	8
Rabbit	60	2
Guinea-pig	60	2
Human	125	4
Heated		
Sheep	30	1
Bovine	60	2
Rabbit	30	1
Guinea-pig	30	1
Human	30	1

Action of spermine on staphylococci suspended in buffer

While some antibacterial agents affect multiplying organisms only, spermine and spermidine have been shown to be bactericidal at 37° and at 6° (Rozansky, Bachrach & Grossowicz, 1954; Rozansky, Razin & Grossowicz, 1954). This killing effect was tested on organisms suspended in a nutrient medium in which even at 6° metabolic activities might still proceed to some extent. The effect of spermine on washed cells suspended in buffer was therefore tested. A 24 hr. broth culture of the staphylococcus was centrifuged, the organisms washed three times and diluted with saline to $1/10^4$; 1 ml. of this suspension was added to 10 ml. of either Sørensen buffer (M/15, pH 7·8) or broth adjusted to pH 7·8. Spermine was added to both series to a concentration of 3×10^{-4} M, and controls without spermine were included. The suspensions were kept at 6° and after the intervals indicated in Fig. 1 the surviving organisms were counted by plating. From Fig. 1 it can be seen that organisms in broth + spermine at 6° died rapidly, with only 2 % surviving after 6 hr. and practically none at 24 hr., but there was almost no bactericidal effect on organisms kept in buffer +

spermine. On prolonged incubation (72 hr.) a decrease of about 50% was noticed in the control tubes and no greater mortality occurred in buffer + spermine.

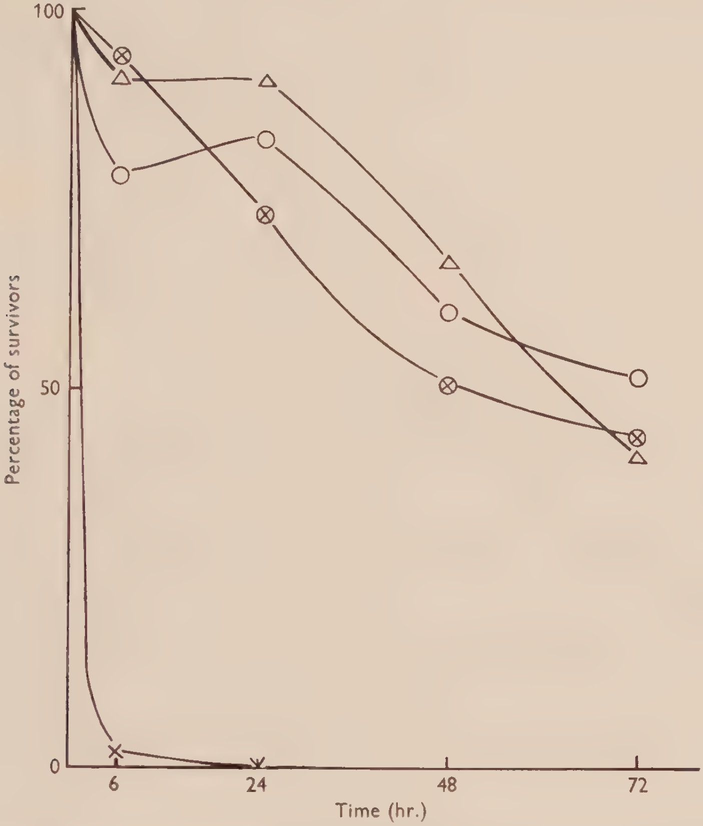

Fig. 1. Effect of spermine on washed cells of *Staphylococcus aureus* at 6°. The pH value of all media was 7·8. The number of surviving bacteria was determined by plate count. Initial inoculum *c.* 10^5 cells/ml. —×—, cells suspended in Difco nutrient broth + spermine.4HCl 100μg./ml.; —⊗—, cells suspended in phosphate buffer + spermine.4HCl 100μg./ml.; —△—, cells suspended in Difco nutrient broth (no spermine); —○—, cells suspended in phosphate buffer (no spermine).

Influence of sodium and potassium chlorides

Spermine and spermidine are bases and act probably at anionic sites on the bacterial cell. The effect of sodium and potassium ions on the action of spermine and spermidine was therefore examined. Increasing concentrations of sodium chloride, potassium chloride, sodium phosphate or sodium sulphate, were made in broth and twofold dilutions of spermine and spermidine were provided. Table 2 shows that the salts antagonized the antibacterial action of spermine and spermidine. A 10-fold increase in the concentration of sodium chloride was accompanied by a 32-fold decrease of spermine, and an 8- to 16-fold decrease of spermidine activity. Potassium chloride antagonized the effects of spermine and spermidine in the same way.

Table 2. *Minimal molar concentrations of spermine and spermidine which inhibit growth of* Staphylococcus aureus *in Difco nutrient broth containing various concentrations of sodium and potassium salts*

The pH value of the medium was 7·7. Size of inoculum: *c.* 10^4 cells/ml. Results were read after 48 hr. incubation at 37°. Inhibition means no visible growth.

	NaCl		KCl		$Na_2HPO_4 \cdot 12H_2O$		Na_2SO_4	
Concentration of salt (%, w/v)	Spermine	Spermidine	Spermine	Spermidine	Spermine	Spermidine	Spermine	Spermidine
	Minimal concentration (M) which inhibited growth							
0·25	$4{\cdot}5 \times 10^{-5}$	$2{\cdot}2 \times 10^{-4}$	9×10^{-5}	$4{\cdot}5 \times 10^{-4}$	$4{\cdot}5 \times 10^{-5}$	$4{\cdot}5 \times 10^{-4}$	$4{\cdot}5 \times 10^{-5}$	$2{\cdot}2 \times 10^{-4}$
0·5	$4{\cdot}5 \times 10^{-5}$	$4{\cdot}5 \times 10^{-4}$	$4{\cdot}5 \times 10^{-5}$	$4{\cdot}5 \times 10^{-4}$	$4{\cdot}5 \times 10^{-5}$	$4{\cdot}5 \times 10^{-4}$	$4{\cdot}5 \times 10^{-5}$	$4{\cdot}5 \times 10^{-4}$
1	$1{\cdot}8 \times 10^{-4}$	$4{\cdot}5 \times 10^{-4}$	$1{\cdot}8 \times 10^{-4}$	$1{\cdot}8 \times 10^{-3}$	$7{\cdot}5 \times 10^{-4}$	$1{\cdot}8 \times 10^{-3}$	$3{\cdot}6 \times 10^{-4}$	9×10^{-4}
2·5	$1{\cdot}5 \times 10^{-3}$	$1{\cdot}8 \times 10^{-3}$	$1{\cdot}5 \times 10^{-3}$	$3{\cdot}7 \times 10^{-3}$	$1{\cdot}5 \times 10^{-3}$	$3{\cdot}7 \times 10^{-3}$	$1{\cdot}5 \times 10^{-3}$	$3{\cdot}7 \times 10^{-3}$

DISCUSSION

Hirsch (1953*a, b*) with *Mycobacterium tuberculosis* found that the antimycobacterial activity of spermine was due, not to the substance itself, but to a derivative of it produced by a thermolabile enzyme, spermine oxidase. This enzyme was present in sheep serum but not in human serum. This enzyme mechanism appears not to operate in the antistaphylococcal action of spermine for the following reasons: (i) The antistaphylococcal tests were performed in an autoclaved medium in which any thermolabile enzyme would have been destroyed. (ii) Spermine and spermidine were active against staphylococci under anaerobic conditions, thus excluding the formation of a derivative activated by oxygen. (iii) Sheep serum was not necessary for the action of spermine and spermidine against staphylococci; on the contrary, serum diminished the antistaphylococcal effect. Sheep and bovine sera exhibited a strong antispermine effect, whereas human, rabbit and guinea-pig sera were considerably less antagonistic. Since the antispermine effect of serum was almost completely destroyed by heating in boiling water for 30 min., it is possible that a protein, perhaps an enzyme, might be responsible for the inactivation of spermine by serum.

Spermine and spermidine are polyamines of pronounced basic reaction and their antibacterial effect may be that of cationic poisons. In this respect they resemble the basic thymus peptide (Hirsch, 1954), polylysine (Katchalski, Bichowski-Slomnitzki & Volcani, 1953) and triethanolamine and similar amines (MacLeod & Onofrey, 1954). The antibacterial effect of spermine and spermidine, and of polylysine and triethanolamine, was antogonized by sodium and potassium. This reversal of spermine toxicity might be visualized as a cation exchange whereby the base spermine is replaced by non-toxic sodium and potassium ions (Massart, 1948). The bactericidal effect of spermine and spermidine was not limited to multiplying staphylococci, but there was no bactericidal action on washed staphylococci suspended in phosphate buffer, i.e. there was a clear difference in susceptibility between resting and non-resting organisms. The non-sensitivity of the organisms suspended in buffer might be due either to their inability to take up spermine or spermidine in the absence of an energy-yielding substrate, or to the absence of some specific metabolic process capable of being blocked by spermine.

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Evidence from the Cytology of *Azotobacter chroococcum* of a Relationship with *Rhizobium* and the Bacillaceae

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SUMMARY: The structure of the cell envelopes in *Azotobacter chroococcum* and the behaviour and mode of formation of the spore-like 'cysts' suggest that this organism has a close phylogenetic relationship with the Bacillaceae, and that accordingly the main genera of nitrogen-fixing bacteria, *Azotobacter*, *Rhizobium*, and *Clostridium*, have a relatively recent common origin.

Cytological studies upon *Rhizobium* spp. (Bisset & Hale, 1951*a*; Bisset, 1952*a*) led to the rather surprising conclusion that this genus is a specialized member of the family Bacillaceae. Further studies upon *Azotobacter chroococcum* (Bisset & Hale, 1953) led to the conclusion (Bisset, 1954) that these two important genera of nitrogen-fixing bacteria have numerous cytological characters in common. The evidence examined at that time included nuclear structures and gonidium-formation, but not, in the case of *A. chroococcum*, a detailed study of the cell envelopes and the characters of the resting-cell, usually called a cyst. It is the purpose of the present paper to show that new evidence strongly reinforces the concept that *Azotobacter* and *Rhizobium* are related, and are specialized members of the Bacillaceae.

METHODS

Seven strains of *Azotobacter chroococcum* were obtained through the courtesy of Mr J. C. Lawrence, by whom they were isolated, in this Department, from soil samples taken in Warwickshire and Somerset. I am also indebted to Mr Lawrence for an indication of the ease with which these strains could be grown upon a medium consisting of 2% (w/v) potato meal with 2% (w/v) agar in tap water. In contrast to most of the recommended media, this simple method produced luxuriant growth in 24 hr. at 25°, thus enabling very young cultures to be studied and compared with much older sporogenous cultures.

The stains employed were: the cell-wall method of Hale (1953); the Victoria blue method of Robinow & Murray (1953); the nitric acid spore stain of Robinow (1951). The latter method is not really a cytological stain, but is rather a test for a diagnostic character of bacterial endospores (Bisset & Hale, 1951*b*).

RESULTS

Cell envelopes

When stained by Victoria blue, the strains of *Azotobacter* examined gave an appearance which can be regarded as reasonably characteristic of the genus, and readily recognizable (Pl. 1, fig. 1). The capsule was clearly visible, the cell wall less obvious, and the cytoplasm in the condition known as 'vacuolated' (Bisset & Hale, 1953). Less deeply stained by the same method, the cells were seen to be subdivided by darker masses representing stages of cross-wall development (Pl. 1, figs. 2, 3). Young cultures stained by Hale's method showed a structure surprisingly like that of typical *Bacillus* spp. (Bisset, 1952*b*) with rods divided by complete cross-walls and subdivided by developing walls (Pl. 1, figs. 4, 5). The development of the cross-walls as an ingrowth was also well seen, in this exceptionally large bacterium (arrows in Pl. 1, figs. 4, 5). By contrast, the same strains, when grown on the chemically defined medium employed by Bisset & Hale (1953), although exhibiting cross-walls, showed a much less Bacillus-like appearance (Pl. 1, fig. 6).

Spores

After a period of 2 or 3 weeks upon potato agar, most of the vegetative cells were transformed into so-called 'cysts'. These resembled spores in being refractile and difficult to stain when mature, although less so when immature. They were also sporelike in being formed within the mother-cell, a state of affairs which is decidedly not suggested by the previous title of cyst (Pl. 1, figs. 7, 8). Under the influence of N-nitric acid the nuclear material of mature spores is partially or completely ejected (Pl. 1, fig. 9). This is suggestive of protoplasmic turgor, and is highly characteristic of the endospores of Bacillaceae (Bisset & Hale, 1951*b*).

The resemblance to true endospores was so complete, from the morphological viewpoint, that it was decided to test their resistance to heat. Five strains were tested; sporogenous cultures, 3 weeks old, were compared with 24 hr. cultures of the same strains. A loopful of culture was emulsified in 1 ml. sterile saline in a screw-capped bijou bottle. These were raised rapidly to 85° in a water-bath, and held at that temperature for 5 min.; the temperature being taken by means of a thermometer in a control bottle. The bottles were then opened, sampled, closed, raised to 95° for 5 min. and re-sampled. Sampling was repeated after heating at 100° for 5, 10 and 15 min.

The samples from the 24 hr. vegetative cultures failed to grow at all stages; all the sporogenous cultures survived 5 min. at 85°; two of these survived 5 min. at 95° and one survived the entire period of the test. This especially resistant spore was also peculiar in being slightly smaller, and a more pointed oval in outline than the others.

DISCUSSION

In addition to the character of nitrogen-fixation, it has already been pointed out (Bisset, 1954) that *Azotobacter* spp. and *Rhizobium* spp. have a number of characters in common. These are known to include: a tendency to Gram-positivity at certain stages in the life-cycle of an apparently Gram-negative organism; the mode of gonidium-formation; the mode of germination of the resting stage; the simultaneous possession of flagella and a mucoid capsule; relatively large size. The Bacillus-like characters of morphology and spore-formation in *Rhizobium* (Bisset, 1952*a*) are now found to be duplicated in *Azotobacter chroococcum*. The cell walls of young cultures upon potato agar have so marked a resemblance to those of the typically large, 'rough' types of *Bacillus* that this alone would give grounds for suspicion of relationship. But the 'cyst' also possesses a number of such endospore-like characters as formation within the mother-cell, refractility, resistance to staining, heat-resistance and nuclear turgor. It is the opinion of the present writer (cf. Bisset, 1950; Bisset & Hale, 1951*b*) that this nuclear turgor, representing some degree of concentration and dehydration of protein, is responsible for the resistant character of bacterial endospores. Neither has so far been found without the other.

If, as is now suggested, the genera *Azotobacter* and *Rhizobium* are both rather aberrant members of the Bacillaceae, these important nitrogen-fixing bacteria are brought into much closer taxonomic connexion with the remaining important group of bacteria which possess this character, the *Clostridium butyricum* group. It was noted that the *Rhizobium* spp. examined by Bisset (1952*a*) most closely resembled the aerogenic megasporous species of *Bacillus*, which themselves resemble *Clostridium* at least as much as they do the typical aerobic species. There is thus evidence obtainable from purely morphological studies which suggests a close connexion between the major groups of nitrogen-fixing bacteria.

A point of cytological interest emerges from the evidence presented here. The large size of *Azotobacter chroococcum* enables the centripetal ingrowth of the cross-walls to be demonstrated quite clearly. This is contrary to my previous belief, but the question had already been resolved by the admirable sections of Chapman & Hillier (1953); the evidence in Pl. 1, figs. 4 and 5, serves merely to confirm their views. On the other hand, the cytoplasmic septa which precede the cross-wall are especially well seen in Pl. 1, fig. 2, and are quite obviously large entire structures, despite the potentially disruptive effect of the 'vacuolated' cytoplasm. Their appearance does not confirm the view of Chapman & Hillier that these septa are an illusion and consist of an unresolved pattern of granules near the ingrowing edge of the cross-wall.

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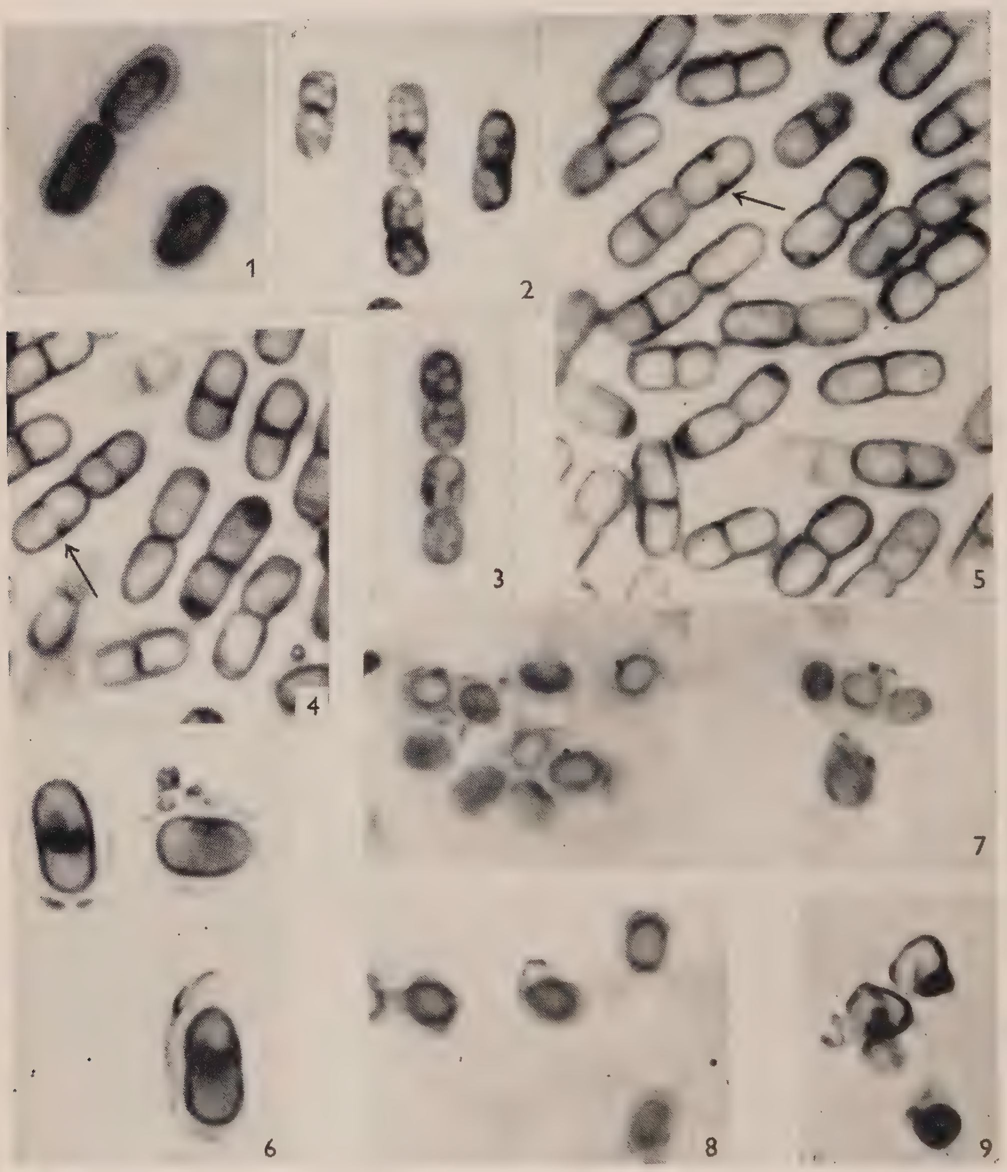

K. A. Bisset—Cytology of *Azotobacter chroococcum*. Plate 1

(*Facing p.* 445)

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EXPLANATION OF PLATE

Figs. 1–3. *Azotobacter chroococcum*, 24 hr. on potato agar. 'Vacuolated' cytoplasm, capsule and cytoplasmic septa seen. Victoria blue. ×3000.

Figs. 4, 5. As figs. 1–3. Cell walls of a distinctly Bacillus-like pattern with centripetally ingrowing cross-walls. Hale's method. ×3000.

Fig. 6. *Azotobacter chroococcum.* 3 to 4-day growth on chemically defined medium. Much less Bacillus-like, but showing well-marked cross-walls. Hale's method. ×3000.

Figs. 7, 8. *Azotobacter chroococcum.* Spores ('cysts') from 3-week cultures on potato agar. Mature spores are refractile and resist the stain; some are enclosed in remnants of sporangium. Hale's method. ×3000.

Fig. 9. As figs. 7, 8. Nucleus ejected under effect of N-nitric acid, crystal violet. ×3000.

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LOMINSKI, I., MORRISON, R. B. & SMITH D. D. (1955). *J. gen. Microbiol.* 13, 446–455

Potentiation and Destruction of R Variant Coagulase by Products of S Variants of *Staphylococcus aureus*

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SUMMARY: Factors obtained from certain S variants of *Staphylococcus aureus* can both increase the potency of and destroy the coagulase produced by R variants of the organism. Evidence that separate factors are responsible for potentiation and for destruction of coagulase is based on differences of pH optimum and of temperature range of the factors. Potentiation of coagulase is most marked below pH 7 and occurs at temperatures ranging from 9° to 37°. The optimum pH value for destruction is between 7 and 8, and it does not occur at 9°. There is evidence, though incomplete, that both factors are enzymes. In the course of both potentiation and destruction the mode of clotting by R coagulase is altered from a granular to a solid type. Some properties of the factors and their partial purification are described and their possible mode of action is discussed.

It has been shown (Smith, Morrison & Lominski, 1952) that high yields of coagulase are obtained from R variants and low yields from S variants of *Staphylococcus aureus*; furthermore, a proportion of the S variants weakly coagulase-positive or coagulase-negative, produce a factor capable of destroying coagulase (Lominski, Smith & Morrison, 1953). The present paper describes the observation that the culture fluids which destroy coagulase can under certain conditions potentiate, i.e. increase the activity of coagulase produced by R variants; an account is also given of some properties of the potentiating and the destroying factors.

There is evidence that potentiation and destruction are due to two different agents, and although complete proof of this is lacking it is expedient to consider them separately. The evidence also suggests that both agents are enzymes; however, again in the absence of definite proof, the word 'factor' is used in preference to enzyme and the agents are referred to as the coagulase-potentiating factor (CPF) and the coagulase-destroying factor (CDF). The terms potentiation and destruction by the factors are used descriptively in a broad sense and do not imply that a simple action of CPF or CDF necessarily takes place.

METHODS

Preparation of coagulase. R variants of either of two strains of *Staphylococcus aureus* (5R and RR) were inoculated heavily into 25–40 ml. volumes of meat extract broth (pH 7·4) in 120 ml. flat bottles and incubated horizontally at 37°. After 4 days the cultures were spun and the cells discarded. Alternatively, 18 hr. growths on sloped agar in similar 120 ml. bottles were suspended, each in 5 ml. of saline, and the cells removed by centrifugation. The coagulase-containing fluid was used, either passed through a Seitz filter or with the addition of 0·05% (w/v) of Thiomersalate (B.D.H.), and after adjustment of the pH to 7 was stored either frozen or at 4°.

Plasma. Human blood-bank plasma containing *c.* 0·4% (w/v) sodium acid citrate and 0·6% (w/v) glucose was used at a final dilution of 1/10 in saline. In all experiments 0·05% (w/v) of thiomersalate and in some experiments also 10 i.u./ml. of heparin (Liquemin, Roche) were added.

Estimation of potency of coagulase. The clotting time was found to be related to the concentration of coagulase within a wide range of dilutions at a given pH value. Accordingly clotting times were used in most experiments as a measure of potency of coagulase. The tests were carried out at room temperature (*c.* 15–20°) in 1 ml. volumes of a mixture of equal parts of coagulase and of 1/5 plasma, in $4 \times \frac{1}{2}$ in. test-tubes. The tubes were tilted through 45° from the vertical every 10 sec. for clotting times under 3 min., then every 15 sec. up to 15 min., every minute for the next 30 min. and every 15 min. up to 6 hr. The clotting times of various coagulase preparations, at a dilution of 1/2, ranged from 30 sec. to 1 min. 30 sec. The potency of coagulase was also estimated in terms of the highest dilution still giving a clot after 24 hr. at 37°; this ranged from 1/2000 to 1/16,000 according to the specimen. The smallest amount of coagulase still giving a clot is referred to as the minimal clotting dose.

Isolation of variants. Coagulase-destroying variants of *Staphylococcus aureus* were isolated as previously described (Lominski *et al.* 1953). Colonies showing on chocolate agar (10%, v/v, horse blood agar heated at 80° for 10 min.) large zones of clearing were first selected; a large number of such colonies were cultured and tested for coagulase destroying activity; those found active were replated and the most active colonies again selected.

Preparation of CPF–CDF. The coagulase-destroying S variants were grown for 18 hr. at 37° on agar slopes in 120 ml. flat bottles, and the growth of each suspended in 3–4 ml. of saline. Alternatively, 40 ml. meat extract broth in 120 ml. bottles were inoculated and either incubated standing at 37° for 4–5 days or shaken (150 oscillations/min.) for 2–3 days at 25°. From both suspensions and cultures the cells were removed by centrifugation and the supernatants used: such material is referred to as CPF–CDF. In some experiments the supernatant fluids were passed through Seitz or sintered glass filters. Unfiltered material was used with 0·05% (w/v) of Thiomersalate. Partial purification was obtained by precipitation with ethanol or ammonium sulphate in the cold.

Estimation of potentiation and destruction of coagulase. Equal parts of coagulase and CPF–CDF were mixed; samples of the mixture were removed at intervals and their clotting times measured. A shortening of clotting time indicated potentiation, and a lengthening destruction of coagulase. Controls consisted of a mixture of coagulase with saline, broth or buffer broth. The tests were made at *c.* 9°, 18–20°, and 37°. The pH values used were from 6·0 to 8·5. When undiluted reagents were used they were adjusted to the required pH value with acetic acid or sodium hydroxide. Any dilutions of the reagents were made in a mixture of equal parts of broth and phosphate buffer (Hendry, 1948).

Quantitative measurement of CPF and of CDF. Dilutions of CPF–CDF were mixed with a constant amount of coagulase and left for 24 hr. at a pH value and temperature appropriate respectively for potentiation (CPF) or destruction (CDF). The clotting times of the mixtures were then measured; the greatest dilution which caused a shortening or a lengthening of clotting time by more than 50 % was taken as the titre.

RESULTS

Production of CPF–CDF

Variants capable of destroying coagulase were isolated from 16 of 20 strains; half were old laboratory cultures and half freshly isolated and all came from human lesions. In some strains the variants were readily found on primary isolation, whereas in others they were seen only after repeated plating. The activity differed considerably from strain to strain and from colony to colony within a variant. Of the strains capable of destroying coagulase four, including the Oxford strain and a variant of the coagulase producing strain 5R, were tested for potentiation and found to be active. Variants of two laboratory strains (KSc and WSc) were found most active and stable, and so were used chiefly as source of CPF–CDF.

The variants grew as smooth medium-sized colonies. They were actively proteolytic (rapid liquefaction of gelatin and peptonization of milk), and on chocolate agar their colonies were surrounded by a zone of clearing. Their phage patterns were reported by Dr R. E. O. Williams to be identical with those of the corresponding R variants.

All but one of the variants were coagulase-positive by the standard test (Fisk, 1940); even the 'coagulase-negative' variant grown at room temperature in the presence of plasma produced a small clot in 2–3 days. However, no coagulase or only traces were present in the CPF–CDF preparations, i.e. in 4–5 day broth cultures or washings of agar slope cultures.

Both CPF and CDF were extracellular. Best and most consistent yields were obtained from agar slopes; broth cultures gave less good results, the best being obtained from cultures shaken at 25°. Increasing the surface of broth cultures exposed to air by horizontal incubation at 37° led to a decrease in both CPF and CDF activities; when bottles were almost full and closed with screw caps only a trace or no CPF–CDF was formed, despite good growth; peptone water cultures (2 and 4 %, w/v) showed either traces or no activity.

So far CPF and CDF have always been found together; numerous estimations of their respective titres carried out in the hope of finding preparations differing in the proportion of the two factors have failed to dissociate them. The CPF–CDF titre measured on dilute coagulase of the same strength under optimum conditions varied from 1/128 to 1/1000 for agar slopes; in broth it was generally lower.

Effect of CPF–CDF on the mode of clotting

With coagulase produced by R variants, the first sign of clotting is the appearance in plasma of fine granules which slowly coalesce and ultimately form a solid clot. When R coagulase has been acted upon for some time by CPF–CDF its mode of clotting is altered; instead of passing through the granular stage there is a sudden increase in viscosity followed almost instantaneously by the formation of a solid opaque clot; this clot formation resembles that obtained with an excess of thrombin. The change in the mode of clotting occurred at all pH values between 6·0 and 8·5, at all temperatures from 9 to 37° and with all coagulase preparations, whether undiluted or diluted coagulase was used, and regardless of the nature of the diluent.

Potentiation of coagulase

In the early stages of the work attention was focused on the destruction of coagulase; the tests were carried out at the natural pH value of the reagents (*c.* pH 7–7·4) at 37° and undiluted coagulase preparations with a short clotting time were used. Under those conditions, a slight decrease in clotting time was occasionally observed shortly after mixing coagulase and CPF–CDF; this was immediately followed by a progressive destruction of coagulase. The shortening of clotting time was at first thought to be an experimental error, and it was not until pH value, temperature and amount of coagulase used were varied that it was realized that in fact two opposite reactions—a shortening and a lengthening of clotting time—were taking place. Further work enabled the conditions to be defined under which potentiation of coagulase is most marked and most easily observed. It was found that potentiation was greatest when dilute coagulase giving a clotting time of more than 4 min. was used (for practical reasons the clotting times were chosen not to exceed 10 min.). Maximum potentiation was obtained only below pH 7; it occurred at all temperatures tested but was fastest at 37°. At temperatures higher than 9°, especially above 20°, potentiation was sooner or later followed by destruction. Table 1 gives the results of a typical experiment.

It can be seen from Table 1 that at pH 6·7 CPF doubled the activity of coagulase (as measured by clotting times) and that at low temperature there was no subsequent destruction. This finding made it possible to confirm by titration the twofold increase in the potency of coagulase. A mixture of coagulase and CPF–CDF, at the time of maximum potentiation, was titrated against plasma under conditions which prevented destruction (pH 6·7, temperature 9°, and undiluted plasma for its inhibitory effect on destruction). Under those conditions potentiated coagulase clotted at a dilution twice as high as the control.

Undiluted coagulase could be potentiated only to a slight degree. It seemed possible that, owing to the increased reaction rate in the presence of large amounts of coagulase the moment of maximum potentiation was being missed. Experiments done at 9° where the reaction proceeds slowly, and using dilute

CPF, showed this not to be the case. It was then thought that, were the reaction between coagulase and CPF a quantitative one, there might not be enough CPF to bring about full potentiation of neat coagulase. Table 2 shows that undiluted coagulase cannot be fully potentiated even when CPF is present in excess.

Table 1. *Effect of* pH *value and temperature on potentiation and destruction of coagulase*

		At pH 6·7								
		9°			20°			37°		
		Clotting times and kind of clot								
Mixture*	Time since mixing	Min.	sec.	Clot	Min.	sec.	Clot	Min.	sec.	Clot
Coagulase 1/10	0	4	45	g	4	30	g	4	30	g
Coagulase 1/20	0	7	0	g	7	0	g	6	45	g
Equal parts of coagulase 1/10 and CPF–CDF 1/9	0	7	15	g	6	30	g	5	45	g
	10 min.	6	45		5	30	f	4	45	s
	20 min.	6	0	f	5	15	s	4	30	s
	30 min.	5	45	s	4	30		5	15	s
	60 min.	5	15	s	—			—		
	80 min.	5	0	s	—			—		
	2 hr.	5	0	s	4	30	s	—		
	3 hr.	—			—			—		
	4 hr.	4	30	s	—			—		
	20 hr.	5	30	s	6	45	s	∞		
Coagulase 1/10	20 hr.	5	45	g	4	45	g	4	45	g
Coagulase 1/20	20 hr.	8	0	g	7	30	g	7	30	g
		At pH 7·6†								
Coagulase 1/10	0	5	0	.	5	0	g	5	0	g
Coagulase 1/20	0	9	0	g	9	0	g	9	0	g
Equal parts of coagulase 1/10 and CPF–CDF 1/9	0	9	0	g	8	45	g	8	30	g
	10 min.	8	45		8	45		8	15	s
	20 min.	8	45		8	30	s	11	0	s
	30 min.	8	15		8	30	s	—		
	60 min.	8	45	s	8	0	s	>20	0	s
	80 min.	—			7	45	s	—		
	2 hr.	—			8	0	s	—		
	3 hr.	7	45	s	9	45	s	>65	0	s
	4 hr.	—			—			—		
	20 hr.	9	30	s	20	0	s	∞		
Coagulase 1/10	20 hr.	5	25	g	5	0	g	5	30	g
Coagulase 1/20	20 hr.	10	30	g	9	25	g	10	10	g

The titre of the coagulase used in this experiment was 18000; the titre of CPF–CDF was 1256.
g, f and s denote granular, flocculent and solid clot. * Concentrations before the addition of plasma.
† At pH 7·6 the clotting times are longer, especially with more dilute coagulase.

The degree of potentiation was found to be independent of the amount of non-coagulase protein present in the coagulase preparation; it was the same with coagulase preparations obtained from broth cultures, saline washings of agar cultures and with coagulase partly purified by the acid, ammonium sulphate and ethanol precipitation method (Tager, 1948*a*). In the majority of experiments the degree of potentiation did not much exceed a doubling in potency. However, preliminary experiments suggest that under certain conditions fractionation of CPF with ethanol may yield preparations which potentiate coagulase up to four times.

Table 2. *Effect of coagulase dilution on degree of potentiation*

pH 6·7; temperature 20°.

Mixture*	Time since mixing	Clotting time (sec.)	Kind of clot
Coagulase	0	68	g
Coagulase 1/2	0	113	g
Equal parts of coagulase and CPF–CDF	0	113	g
	2 min.	105	f
	4 min.	104	f
	8 min.	102	s
	10 min.	105	s
	35 min.	145	s
	2 hr.	160	s
	3½ hr.	175	s
	20 hr.	710	s

Mixture*	Time since mixing	Clotting time min.	Clotting time sec.	Kind of clot
Coagulase 1/8	0	5	15	g
Coagulase 1/16	0	8	0	g
Equal parts of coagulase 1/8 and CPF–CDF 1/20	0	7	45	g
	40 min.	5	30	f
	50 min.	5	30	s
	90 min.	5	15	s
	2 hr.	4	45	s
	—		—	
	—		—	
	—		—	
	20 hr.	5	30	s

* Concentrations before the addition of plasma. g, f, s denote granular, flocculent and solid clot. In these experiments the clotting times are longer than in Table 1; a different batch of coagulase and of CPF–CDF was used.

Properties of potentiated coagulase

A coagulase preparation on which CPF had acted differed from the original coagulase preparation, not only in its mode of clotting, but also in that its clotting time could not be shortened again by the addition of more CPF.

Other things being equal, the amount of coagulase surviving heating depends on the initial potency of the preparation. The heat sensitivity of potentiated coagulase was therefore compared with that of untreated coagulase. The loss of activity, on heating at 80° at pH 7, for periods of time from 5 to 20 min. was found to be identical with potentiated coagulase and untreated coagulase of the same clotting time.

The reaction rates of potentiated coagulase and of untreated coagulase with ‘activator’ (Smith & Hale, 1944) were also investigated. The activator was prepared by a slight modification of the ammonium sulphate method

(Tager, 1948*b*). It was found that potentiated coagulase reacted with activator at the same rate as untreated coagulase of the same clotting time and formed ultimately the same amount of 'activated coagulase'.

Destruction of coagulase

With both undiluted and diluted coagulase, destruction was shown by a lengthening of clotting time which progressed to a total disappearance of clotting power; with undiluted coagulase the destruction rate was greater. Destruction was fastest at temperatures above 20° and a pH value above 7: when both pH and temperature were low, destruction was negligible. Below pH 7·6 and especially at temperatures lower than 37° destruction was preceded by some degree of potentiation. Table 1 shows destruction at 20° and 37°, and pH values of 6·7 and 7·6.

Destruction of coagulase was also demonstrated by titrating the amount of coagulase left after interaction with CDF. This was done by altering the pH value from 7·6 to 6·7 and carrying out the titration at 9° on undiluted plasma. The results obtained in this way confirmed those of clotting times.

Role of plasma

With occasional specimens of plasma potentiation of coagulase could not be fully demonstrated. All the samples in question inhibited untreated coagulase and gave long clotting times when tested with a standard preparation.

Properties of CPF–CDF

Strain specificity. CPF–CDF produced by variants of all four strains potentiated and destroyed coagulase produced by the two R variants.

Filtrability. CPF–CDF passed without loss of activity through Seitz filters and sintered glass filters (0·9 μ.).

Heat resistance. Heating at 100° for 5 min. decreased the activity of both factors to about half the initial value; heating for 20 min. left approximately 5–10 % activity.

Inhibition. Both CPF and CDF were considerably but incompletely inhibited in the presence of whole human, ox serum or 'refined' globulin preparations (of diphtheria and *Clostridium welchii* antitoxic sera), but not by the soya-bean trypsin inhibitor (Kunitz, 1945–6).

Temperature and pH *optimum.* The value of the data in these respects is necessarily restricted since in most experiments the two reactions of potentiation and destruction were bound to have run concurrently. The action of CPF was approximately 12 times faster at 37° than at 9°. CDF activity increased slowly with increase of temperature and there was a strikingly sudden increase of activity between 20° and 37°; higher temperatures were not tested. At 9° there was no evidence of CDF activity.

The optimum pH value for CPF lay between 6 and 7; the optimum for CDF between 7 and 8. The curve of activity of CDF showed a steeper fall on the acid side than on the alkaline side but some destruction took place even at pH 5·6.

Partial purification of CPF–CDF

Saline washings from agar slopes were used as starting material. By ammonium sulphate precipitation maximum activity was found in the 60 % and the 70 % saturation fractions. With ethanol (pH of material 7, precipitation for 12 hr. at 8°) the maximum activity was in the fraction between 40 and 75 % (v/v); the fraction redissolved in the original volume of saline showed half the activity of the crude material but its nitrogen content was decreased from 0·494 mg./ml. to 0·007 mg./ml. Separation of CPF from CDF by either ammonium sulphate or ethanol precipitation was not achieved.

The nature of CPF and CDF

An attempt was made to examine whether potentiation and destruction are enzymic processes by comparing the activity of CPF–CDF which had acted on several successive additions of coagulase, with that of a control preparation diluted to the same extent. CPF–CDF was allowed first to potentiate and then to destroy 2000 minimal clotting doses of coagulase at pH 6·7 and at a temperature of 20° for potentiation, and at 37° for destruction; this was repeated three times. On the fourth addition the rate of potentiation and destruction was identical with that of the control preparation of CPF–CDF brought to the same dilution.

In another experiment potentiation was allowed to proceed to its maximum at pH 6·7 at 20°; the material was then diluted 40 times with buffer broth pH 6·7 (thus minimizing the effect of coagulase present on clotting time) and used for potentiation of a fresh lot of coagulase. The rate of potentiation was again found identical with that of the control preparation of CPF–CDF similarly diluted. These observations support the idea that CPF and CDF may be enzymic in nature.

The distinction of CPF–CDF from gelatinase and casein protease

Variants which produce CPF–CDF appear on the whole strongly proteolytic (much more so than the corresponding R variants). They liquefy gelatin and peptonize milk rapidly; the zone of clearing formed around their colonies on chocolate agar is presumably also due to proteolysis. CPF–CDF preparations attacked casein, globulins, peptones, fibrinogen and gelatin with liberation of ninhydrin-positive material. Attempts to separate CPF–CDF from the proteolytic enzymes present in the material by fractionation failed. It proved possible, however, to distinguish between CPF–CDF and gelatinase: two variants were encountered, one of which was highly gelatinolytic with no action on coagulase; the other yielded in broth cultures no gelatinase but CPF–CDF to a high titre. Also CPF–CDF was distinguishable from casein protease: cultures in peptone water had little or no action on coagulase but peptonized milk at a dilution of 1/16. Conversely, broth cultures of the same variants, active on coagulase, were inactive on milk.

Action of trypsin on coagulase

It is known that trypsin destroys coagulase (Tager, 1948*a*); therefore an attempt was made to see whether trypsin could potentiate coagulase. At concentrations of trypsin from 1/1000 to 1/60,000 (w/v) and pH values ranging from 6·7 to 7·6 only a progressive lengthening of clotting time and no shortening was observed; trypsin, however, altered the mode of clotting of R coagulase in the same way as did CPF–CDF preparations.

DISCUSSION

The difference in pH optimum and in the range of temperatures at which potentiation and destruction of coagulase occur suggest that CPF and CDF are two distinct factors. It is possible to suppose the continuous action of one factor might account for both phenomena, but to assume two factors has meanwhile the merit of simplicity. Of course, it must be borne in mind that possibly neither CPF nor CDF is a single substance. The fact that the reaction rates of CPF and CDF appear unaffected by previous interaction with coagulase makes a simple stoichiometric reaction unlikely; along with the inhibition by globulins it renders the enzymic nature of both factors probable.

The present study yields no information as to the mechanism of the change of mode of clotting shown by R coagulase after CPF–CDF or trypsin have acted upon it. Ferry & Shulman (1949) and Edsall & Lever (1951) showed that the character of thrombin clot is affected by the presence of acetyltryptophanate, urea, guanidinium chloride and various hydroxyl compounds. A similar mechanism may operate in the case of coagulase.

The shortening of clotting time, the increase in titre, the heat resistance and the rate of reaction of potentiated coagulase with activator suggest that under the action of CPF apparently more coagulase becomes demonstrable. The possibility of the converse, namely that coagulase forms more coagulase from CPF may, we believe, be discarded since the clotting time of potentiated coagulase depends on the original amount of coagulase present and not on the concentration of CPF.

Only a very hypothetical picture of the mechanism of potentiation can be offered. Potentiation may be regarded as the result of the action of CPF either on a substance other than coagulase or on coagulase itself. The increase in potency could be the result of the removal of an inhibitor or of the formation of an accelerator; here, the accelerating action of protamine and polylysine on coagulase clotting has been recently reported (Biezunski, Shafrir, De Vries & Katchalski, 1955). The appearance of fresh reactive groups or the splitting of the coagulase molecule into smaller but still active fragments could also be visualized.

It is difficult to understand why undiluted coagulase, even under optimum conditions, cannot be potentiated to the same degree as dilute coagulase; it is not due to lack of an adequate amount of CPF since this was shown to be present in excess. Inhibition by either the menstruum or potentiated coagulase could account for this.

Assuming that CDF is an enzyme, the destruction of coagulase may be considered to be the result of proteolysis. The phenomenon is reminiscent of the destruction of the antigenic M protein of haemolytic streptococci by their own protease (Elliott, 1945). The detection of coagulase has now assumed a prime significance in the identification of *Staphylococcus aureus*; considerable importance has also been attributed to coagulase in the pathogenesis of staphylococcal infections (Hale & Smith, 1945; Smith, Hale & Smith, 1947). It is tempting to think that CPF and CDF may not only occasionally play havoc with the recognition of pathogenic staphylococci *in vitro*, but also have a role *in vivo*.

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A Selective Medium for *Bacillus anthracis*

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SUMMARY: A medium containing propamidine is described which has high selective activity for the species *Bacillus anthracis*. The spore form of the organism is essential as inoculum for the medium.

The isolation of *Bacillus anthracis* from material heavily contaminated by other organisms of the same genus can be difficult, particularly when small numbers of *B. anthracis* are present, or when quantitative examination is required. Pearce & Powell (1951) did much to simplify this problem by devising a medium containing lysozyme and haemin, but considerable numbers of other sporing organisms found in soil were not suppressed. The antibacterial activity of diamidines has been studied by many workers (e.g. Bernheim, 1943; Elson, 1945; Wein, Harrison & Freeman, 1948). Lacey (1954) used 4 : 4-diamidino-phenylamine as a selective agent for *Haemophilus pertussis*. In the present study it was found that 4 : 4-diamidino-diphenoxypropane (propamidine) allowed the germination of *Bacillus anthracis* spores with subsequent growth and multiplication of cells while it inhibited the growth of practically all other organisms tested. The medium finally devised is more selective for *B. anthracis* than any other so far reported.

METHODS

Media. The basal medium was a peptone agar containing Evans peptone 2 % (w/v), NaCl 0·5 % (w/v) and agar 2 % (Kobé no. 1; Stafford Allen and Sons Ltd.) adjusted to pH 7·6 and sterilized at 120° for 20 min. For control purposes this medium was used with and without the addition of 1 % (v/v) packed red blood cells (several different species were used, see below). The test medium contained 1 % packed red cells, 0·01 % (w/v) propamidine isethionate (May and Baker Ltd.) and 20 units polymyxin B ('Aerosporin' Burroughs Wellcome and Co.)/ml. The additions were made to the melted agar cooled to 50° and poured into Petri dishes in 20 ml. amounts. The agar plates were dried for 2 hr. at 37° to remove surface moisture and could be stored at 4° for at least 3 weeks without loss in selective properties.

Human, horse, sheep, or rabbit blood cells could be used. Whole blood was as good as packed cells, but concentrations above 2 % (v/v) allowed the growth of many more contaminants present in the inoculum.

Changes in the pH value of the medium greatly affected the inhibitory activity of the propamidine. The higher activity at pH 8·0 restricted the growth of *Bacillus anthracis*, while at pH 7·0 the medium had a diminished selective action.

Growth tests. The Miles & Misra (1938) technique was used to inoculate the control and test media. Broth cultures, spore suspensions and soil extracts were tested. The latter were prepared by extracting various samples of soil to which *Bacillus anthracis* had been added with water (1 g. soil/20 ml. water) and heating at 60° for 30 min. before use. Portions of the heated soil extract were also filtered through Millipore membranes (Millipore Filter Corporation of America) which after filtration were placed with deposit upwards on the agar media.

Air from animal rooms and laboratories was sampled directly upon the control and test agar plates with the slit sampler of Bourdillon, Lidwell & Thomas (1941).

In all tests incubation was at 37° for 18 hr.

RESULTS

Growth of pure cultures. Seven species of spore-forming aerobes (in all 40 strains) and 7 species of non-spore-forming aerobes (21 strains) were tested. The 15 strains of *Bacillus anthracis* examined all gave quantitatively similar counts on the propamidine and control agar. Colonies were slightly smaller (1·8 mm.) on the test medium than on the control agar (2·0 mm.), and the 'medusa head' effect more marked. There was no difference between the morphology of organisms from test and control media.

The other spore-forming aerobes examined were completely inhibited on the propamidine medium, with the exception of some strains of *Bacillus cereus.* Most strains of this species, however, were readily differentiated from *B. anthracis* by their production of marked haemolysis in the medium and by their larger colony size (3–4 mm.).

The other Gram-positive and Gram-negative species tested were completely inhibited with the exception of *Proteus vulgaris.* This organism was prevented from swarming, and the growth of one strain was markedly decreased. Results are summarized in Table 1.

Tests with soil extracts. Many samples of soil from downland and farmyards in Wiltshire were examined. There was great variation in number and type of organisms from one sample to another, and a detailed description of results would be of little value for the present purpose. Growth of the undiluted soil extract (0·2 ml./plate) on the control medium was generally confluent, but the highest number of colonies, other than *Bacillus anthracis*, encountered on the propamidine agar was 35/plate. The majority of these colonies were immediately differentiated from those of *B. anthracis* by their colonial appearance, but some non-haemolytic strains of *B. cereus* required incubation for at least 24 hr. before their colonies could be differentiated with certainty. A small number of *B. mycoides* (*B. cereus* var. *mycoides*) strains were grown but they gave nearly invisible colonies on the propamidine medium.

Tests with Millipore filters. When 5 ml. amounts of soil extracts were filtered through Millipore pads of 6 cm. diameter, and the deposit on the pads cultivated as previously described, there was occasionally an overgrowth of

Bacillus anthracis by *B. cereus*. *B. anthracis* was isolated, however, when 50 spores were added to 1 g. soil and 1–3 ml. amounts of the extract were passed through similar Millipore filters. The immediate identification of *B. anthracis* colonies which developed on the filter pads was difficult since they were smaller (1·5 mm.) and had a smoother appearance than when grown directly on the agar surface.

Table 1. *Growth tests of various organisms on the propamidine medium*

Organism		Percentage of control count
Bacillus anthracis	NCTC 5444, 8234, 2620, 4991, 1712, 1711, 1328, 109	100
B. anthracis	(7)	100
B. cereus	NCTC 8035	100
B. cereus	(6)	Nil–100
B. subtilis	NCTC 3610	Nil
B. subtilis	(6)	Nil
B. '*mycoides*'	(4)	Nil
B. licheniformis	(3)	Nil
B. megaterium	(2)	Nil
B. pumilus	(2)	Nil
Proteus vulgaris	(3)	30–100
Escherichia coli	(4)	Nil
Aerobacter aerogenes	(2)	Nil
Alcaligenes faecalis	(6)	Nil
Streptococcus faecalis	(1)	Nil
Micrococcus tetragenus	(3)	Nil
Staphylococcus aureus	(2)	Nil

Figures in parentheses refer to the number of laboratory strains tested. Spore suspensions were used where applicable.

Tests with air samples. The propamidine medium inhibited 90% of the organisms which appeared on control plates. The colonies of those organisms which grew were minute and would readily have been differentiated from *Bacillus anthracis* had it been present in the air sampled.

Inhibition of vegetative cell inocula. It was found that while an inoculum of *Bacillus anthracis* spores was not inhibited on the propamidine medium, a vegetative cell inoculum from broth culture was markedly affected. This phenomenon persisted with dilution of the propamidine to the point where no selective action on other organisms occurred. The results of a typical experiment are summarized in Table 2. Flasks of broth (basal medium without agar) and of broth + 0·005% (w/v) propamidine were inoculated with *B. anthracis* spores so that a measured sample from the flasks would contain *c.* 200 spores. Such samples were taken before and during germination of the spores and plated on control and propamidine agar.

It is seen from Table 2 that the vegetative organisms from *Bacillus anthracis* spores which had germinated in the presence of propamidine were able to grow normally on the propamidine agar, while vegetative organisms from spores which had germinated in the control broth were markedly inhibited when plated on propamidine agar.

In similar experiments with *Bacillus subtilis* and *B. cereus* (Table 3) samples from the germination broths were also heated at 60° for 30 min. to establish whether germination of the spores was affected by propamidine. The results in Table 3 show that although germination of spores of both species was normal in the presence of propamidine, *B. subtilis* spores were unable to acquire resistance to this substance. *B. cereus*, however, was naturally resistant to propamidine and vegetative organisms from spores which had germinated in control broth developed normally on the propamidine medium.

Table 2. *Growth from* Bacillus anthracis (M36) *spores germinated in peptone broth or in propamidine broth*

Germination medium	Period of incubation (hr.)	Counts of broth samples plated on: Propamidine agar	Control agar
Peptone broth	0	195	210
	1	75	195
	2	48	213
	4	24	513
Peptone broth + 0·005 % (w/v) propamidine	0	201	207
	1	225	207
	2	222	216
	4	441	458

Table 3. *Growth of* Bacillus cereus *and* B. subtilis *spores germinated in peptone broth or in propamidine broth, and then plated on control agar or propamidine agar*

Organism	Germination medium	Period of incubation (hr.)	Counts of broth samples plated on: Propamidine agar	Control agar	Counts of heated broth samples* plated on: Control agar
B. cereus NCTC 8035	Peptone broth	0	168	175	151
		1	165	153	0
		2	320	312	0
		4	TNTC	TNTC	0
	Peptone broth + 0·005 % (w/v) propamidine	0	151	178	147
		1	185	163	0
		2	364	381	0
		4	TNTC	TNTC	0
B. subtilis NCTC 3610	Peptone broth	0	0	240	216
		1	0	168	0
		2	0	149	0
		4	0	2400	0
	Peptone broth + 0·005 % (w/v) propamidine	0	0	253	241
		1	0	191	0
		2	0	95	0
		4	0	41	0

* Held at 60° for 30 min. before plating; TNTC, too numerous to count.

DISCUSSION

The induction of resistance to the antibacterial action of propamidine in *Bacillus anthracis* has been shown to be a characteristic of the metabolic activity of the spore germinating in the presence of this substance. Vegetative organisms grown in the absence of propamidine fail to develop when placed in its presence. In the usual attempts to isolate *B. anthracis* from contaminated material such as soil, the spore form is always encountered. Inhibition by the propamidine medium of the vegetative form of *B. anthracis*, therefore, is not a serious disadvantage. The vegetative forms of other species, with the exception of *B. cereus*, appear unable to acquire resistance to propamidine when the spores germinate in the presence of a high concentration of propamidine.

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Genetic Studies on Small-colony Variants of *Escherichia coli* K-12

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SUMMARY: Stable small-colony variants of *Escherichia coli* K-12 and several of its auxotrophs were isolated by treatment of cultures with copper sulphate solutions. These variants did not show any associated changes in nutritional, fermentative or serological characters. The small-colony forms showed normal recombining character in two instances, but in one variant the F− form showed a lower recombination rate and atypical transfer of markers. However, when rendered F+ this variant behaved in a manner analogous to the normal F+ parent strain. The results have been interpreted as evidence that the small-colony character can arise by a nuclear mutation at one of several possible genetic loci.

The isolation of variants which differed from the parent strains by exhibiting a smaller colonial size on solid media has been reported by many authors (Braun, 1947) from a variety of species of bacteria. Most variants were isolated from unfavourable environments produced by antibiotics, toxic inorganic salts or by ageing, and in many instances concomitant changes were found in nutritional requirements, fermentative capacities and serological types. Kuhn & Sternberg (1931) showed that small-colony variants (SCVs) could readily be isolated from a variety of widely differing bacterial species by the use either of ammonia or by transient exposure to phenol. Interest in these small- or 'dwarf'-colony variants was greatly stimulated in 1931 by the report by Hadley, Delves & Klimek that small-colony variants isolated from *Shigella* spp. by the use of lithium chloride passed through bacteriological filters. It was suggested by these authors that the variants represented a stage in the development cycle of the vegetative growth of the bacterium for which the term 'gonidial' or G form was proposed. Moreover, these G forms were reported to differ in nutritional, fermentative and serological properties from the original strain. Also using LiCl, Swingle (1934, 1935) isolated small colonies from *Staphylococcus aureus*, but she was unable to demonstrate the filterability of these variants. Although they had apparently lost certain fermentative capacities, they were antigenically unchanged. The loss in fermentation capacity was verified by Youmans (1937) by the use of the same salt on *S. aureus*. He suggested that these loss variations were due to an overall lowering of the metabolic rate of the small-colony variants, produced in response to the inhibitory environment. By the use of 2-methyl-1 : 4-naphthoquinone (menaphthone) Colwell (1946) was able to isolate stable small-colony variants of *Escherichia coli* which resembled those of *Staphylococcus aureus* already reported, both in their frequent reversion to normal size in liquid media and also by their loss of certain fermentation capacities. Voureka (1951), by the use of chloramphenicol and specific antiserum, isolated from *Escherichia coli* a small-colony variant which differed from the parent strain only in its antibiotic sensitivities. Manten & Rowley (1953) produced small colony variants of *E. coli* by the use of chloramphenicol and normal serum, and showed that in most instances no change had arisen in any other character. When *Staphylococcus aureus* was treated with the same antibiotic under conditions which excluded the selection of spontaneous mutants (Voureka, 1952), dwarf variants were produced. The decrease in colonial size was associated with losses in biochemical activities, phage sensitivities and mouse virulence but not with sensitivity to chloramphenicol. Weed & Longfellow (1954) reported the isolation of small-colony forms of *Escherichia coli* after treatment with low (5×10^{-6} M) concentrations of cupric ion, and Wise & Spink (1954) have isolated small-colony variants from *Staphylococcus aureus* by the use of various antibiotics *in vitro*, and from human cases with a history of antibiotic therapy.

Although Youmans (1937) has suggested that loss in nutritional and fermentation characters is related to a lower metabolic rate in the small colonies, concomitant qualitative changes of serological and phage relationships reported by various authors are difficult to account for by the concept of a single gene mutation. Ephrussi (1953) showed that in *Saccharomyces* spp., small-colony variants, which he called 'petites', can arise as a result of a loss of cytoplasmic genetic units. It was of interest therefore to attempt to isolate small-colony forms from bacterial strains that undergo genetic recombination. Recombination studies of such variants should help to clarify certain of the conflicting hypotheses that have been proposed to account for this phenomenon (Braun, 1947).

METHODS

Bacterial strains. The K-12 strain of *Escherichia coli* and several of its well-investigated auxotrophic mutants 58-161 (requiring methionine), and W-677 (requiring threonine, leucine and thiamine) were used both for the isolation of small-colony variants and in subsequent recombinations. F+ and F− types (Hayes, 1953) of streptomycin-sensitive and of streptomycin-resistant mutants of each auxotroph were used.

Minimal medium (MM) of Davis & Mingioli (1950) was used for the isolation of small-colony variants or in some instances the basal medium of Weed & Longfellow (1954), both supplemented with appropriate growth factors where necessary.

Nutrient media of beef digest, veal digest, blood, boiled blood and MacConkey agar were those routinely used in this Institute.

Partially-defined complete media used were those of Cohen & Wheeler (1946) solidified with agar and with added charcoal and catalase (Mazloum & Rowley, 1955), eosin methylene blue sugar (EMB) medium (Lederberg, 1950) solidified with either agar or silicic acid (Taylor, 1950) and a peptone Lemco Yeastrel Pronutrin (PLYP) medium quoted by Challice & Gorrill (1954). Eosin methylene blue (EMB) agar (Lederberg, 1950) was used for the scoring of sugar fermentation characters.

Recombination techniques employed were the standard auxotrophic and streptomycin prototrophic techniques of Lederberg (1951), and were carried out as described previously (Clowes & Rowley, 1954*b*).

Transfer of the F+ character was achieved by incubation in fresh broth (10 ml.) of a mixture of overnight cultures of a St^S F+ strain (0·2 ml.) and a St^R F− strain (0·01 ml.) for 18 hr. at 37°. Subsequent plating on streptomycin nutrient agar permitted growth of the St^R strain, separate colonies of which were tested for the F+ character by mating with a complementary F− strain.

Sugar fermentation markers were scored on plates of EMB agar by the velveteen replica plating method of Lederberg & Lederberg (1952). The master plate used was prepared by emulsifying the recombinant colonies in saline and inoculating on a plate of the selective medium positioned above a template.

Colonial size of the recombinants was tested in the following manner. Inocula of colonies from a replica of the recombination plate were streaked on the same nutrient agar plate as control streakings of both parents taken from cultures on solid media inoculated at the same time as the replication.

Growth curves were plotted from cultures in nutrient broth and in minimal media. Overnight cultures were diluted to give *c.* 100 cells/ml. and incubated at 37° with shaking in 10 ml. samples in bottles which could be accommodated in a Hilger photoelectric spectrometer (Spekker). Viable cell counts were made by taking samples at hourly intervals, serially diluting in saline in duplicate and inoculating 0·02 ml. drops on the surface of nutrient agar plates (Miles & Misra, 1938). When the growth had reached visible opalescence, parallel turbidity estimations were made in the 'Spekker'.

Motility measurements were carried out by determining the penetration of a motile strain along a column of sloppy agar. The apparatus, shown in Pl. 1, fig. 1, consists of a series of capillary tubes of 1 mm. bore and 45 cm. long. These were drawn out at one end, which was treated with silicone, and the other end was joined to a 3 cm. length of 4 mm. bore soft glass tube. After heat sterilization, sterile molten nutrient agar (Difco 0·4 %) was drawn up the tube to form an uninterrupted column of agar about 35 cm. long. A 4 hr. broth culture of the motile strain, previously passed through a Craigie tube, was diluted (1/100) and *c.* 0·02 ml. inoculated to make contact with one end of the solidified sloppy agar column, which was allowed to extrude slightly from the capillary into the wider end of the tube. The inoculum was then drawn into the capillary by allowing the agar to move towards the drawn-out end, so that finally the agar column and inoculum occupied a position midway in the capillary. The tubes were incubated horizontally for 18 hr. at 37°. The drawn-out silicone end was then sterilized by flaming, and by means of a perforated teat the column was extruded in measured lengths into 2 ml. samples of nutrient broth. The extent of movement of the motile organism down the capillary was then found by the end-point of growth in the samples of broth after incubation.

It was found that under standard conditions triplicate assays always gave results within 5 cm. Young broth cultures gave the fastest speeds; these decreased with the age of the cultures, and 4–6 hr. cultures were normally used in determinations. Variation in inoculum size from 0·02 ml. of an undiluted 4 hr. broth culture down to 0·02 ml. of a culture diluted 1/1000 gave no variation in results, but further dilution below 1/1000 (corresponding to *c.* 500 cells) decreased the movement. Increase in agar concentration decreased movement, 0·4 % being the concentration chosen to give sufficient rigidity to the agar column, whilst allowing maximum movement of fastest strains after 18–24 hr. incubation at 37°. Controls of non-motile *Escherichia coli* B showed no movement (Furness & Rowley, 1955).

RESULTS

Isolation of small-colony variants

Attempts to isolate stable small-colony forms of *Escherichia coli* K-12 or its auxotrophs by the techniques of Colwell (1946) and Voureka (1951) were without success. By the technique of Weed & Longfellow (1954) stable variants were isolated with relative ease from all strains, although the inhibitory concentration of cupric ions was found to be higher in the case of *E. coli* K-12 (10^{-4} M) than with *E. coli* B ($2{\cdot}5 \times 10^{-3}$ M). In detail the method consisted of the addition of 10^8 cells of a fresh culture of the strain to 25 ml. volumes of MM medium (Davis & Mingioli, 1950) containing copper at concentrations of 2×10^{-4}, 10^{-4}, 5×10^{-5} and $2{\cdot}5 \times 10^{-5}$ M, which were then incubated at 37°. Samples were plated on nutrient agar after 24 hr. and 30 hr. The cultures at the two lowest concentrations of copper usually showed turbid growth after 24 hr., and the majority of the colonies isolated were of normal size; the culture containing 10^{-4} M copper showed little visible turbidity but on plating, between 10 and 50 % of the colonies were found to be small. From the highest concentration of copper small colonies were isolated, but the cultures were frequently sterile after 24 hr. incubation. On subculture 98–99 % of all the small colonies tested reverted to the normal size, the remainder retaining the small colonial appearance. Thus two small-colonies designated K-12–1, K-12–2 were isolated in separate experiments from *E. coli* K-12 cultures in 5×10^{-5} M copper, one colony designated W-677–7 from the incubation in $2{\cdot}5 \times 10^{-5}$ M copper of W-677/St^R/F – and two colonies 58–161/1 and 58–161/2 from the same culture of 58–161/St^R/F – in medium containing 2×10^{-4} M copper. The growth of K-12 and K-12–1 spread on the same nutrient agar plate after 18 hr. incubation at 37° is illustrated in Pl. 1, fig. 2.

Properties of small-colonies

The small-colony variants showed colonial diameters of 0·75–1·0 mm. after incubation for 18 hr. at 37° on nutrient agar, as compared with a diameter of 2–3 mm. shown by the parent strains under these conditions. They may therefore be different from the relatively smaller forms isolated from *Escherichia coli* by Colwell (1946) and by Weed & Longfellow (1954), whose variants had colonial diameters of a sixth to a tenth those of the original strains. The present small-colony variants conform to the relative size of the majority of variants isolated by Manten & Rowley (1953) by the use of chloramphenicol and serum, and also to the 'petites' of Ephrussi (1953). In addition, the variants, without exception, resembled the parent strains in all other characters tested, including methyl-red, Vosges-Proskauer and indole reactions, sugar fermentations, sensitivities to coliphages T_1 to T_7, auxotrophic requirements and inhibitions by amino acids (Rowley, 1953); and they were agglutinated by *E. coli* K-12 rabbit O antiserum to the same titre as the parent strain (1/6400). In this way they resembled the stable variant isolated by Voureka (1951), but differed from the small colonies of *E. coli* of Colwell (1946), Weed & Longfellow (1954), and from those of other species investigated by many authors who found accompanying decreases in certain fermentative and synthetic activities of the original strain.

When the variants derived from F− strains were tested they were found to have retained the F− recombining character, in that they mated only with F+ strains. They could be rendered F+, the small colonial character being preserved.

The colonial morphology of all small-colony variants was characteristic of smooth *Escherichia coli* colonies of that size. Morphologically they were indistinguishable from the parent strain, although on first isolation stained smears revealed pleomorphic rods in addition to coccobacillary and coccoid forms as described by Voureka (1951). This pleomorphism was characteristic of the whole culture in the presence of inhibitory concentrations of chloramphenicol or copper.

So far we have not been able to account for the small colonial size. That lack of a growth factor was responsible seemed possible from the ability to vary the ratio of the small to normal colonial size by growth on various solid media. However, none of the media used, which included blood, boiled blood, minimal, nutrient, EMB, veal digest, PLYP and MacConkey agar, all with or without a supplement of 1% glucose, permitted the small colonies to grow to more than half the size of the colonies of the corresponding normal strain. Furthermore, on MM medium + Casamino acids, *p*-aminobenzoic acid, yeast nucleic acids, glutathione, thiamine, pantothenic acid, riboflavin, nicotinamide, or pyridoxine either individually or in various mixtures, there was no change in the relative sizes of the SCVs and parental colonies. The small-colony variants do not, therefore, appear to be deficient in certain synthetic abilities as are those variants described by Weinberg (1950) and Gillespie (1952).

The absence of a self-inhibitor such as is produced by *Haemophilus pertussis* (Mazloum & Rowley, 1955), was shown by the maintenance of the small colonial size on Cohen & Wheeler medium supplemented with charcoal and catalase. The preservation of colonial size ratios on plates of EMB medium solidified with silica gel (Taylor, 1950), indicated that an intolerance to some inhibitor present in agar was not responsible.

Lack of analogy with the 'petite' colonies isolated by Ephrussi (1953) from yeast was shown by the maintenance of the colonial size ratios under anaerobic conditions of growth on nutrient agar, when the colonies of both parental and small colonial strains were diminished to about a third the size of those obtained under aerobic conditions. Growth in air + 5 % or 10 % (v/v) CO_2 did not alter colony sizes, indicating that the variants did not arise by interference in the pyruvic acid decarboxylation system as was found by Hale (1951) in the case of certain G variants of *Staphylococcus aureus*.

Differences in colonial size in *Escherichia coli* have been accounted for in certain instances by variations in polysaccharide production (Wilkinson, Duguid & Edmunds, 1954). It seemed possible that the parent and variant colonies might contain the same number of cells but of different size because of a diminished extracellular secretion of some metabolic product by the small colonies. Viable counts of whole colonies made in triplicate by the Miles & Misra (1938) drop technique, however, indicated that the numbers of cells in the large parental colonies were 20–50 times as great as in the variants, and excluded this possibility.

Electron micrographs taken of the colonies on nutrient agar, by the 'pseudo-replica' method of Hillier & Baker (1946) on to sheets of collodion, failed to show any differences in the arrangement or packing of the cells within the colonies that might have been related to colonial size.

When small-colony variants were inoculated on plates of sloppy (1 %) nutrient agar, growth spread across the plate, and an examination, by the hanging-drop technique, of cells taken from the periphery of growth, showed the presence of motile cells. The parental strains were motile under the same conditions. However, in the case of two of the variants (K-12–1, W-677–7), the time taken to spread across the plate was longer than that taken by the parental strains, this speed of movement being a constant factor which was not altered by successive subculture from the periphery of growth. Measurements of the speeds of movement of the small-colony variants and of the parental strains were made by determinations of the penetration of the organisms along columns of 0·4 % nutrient agar; the results are summarized in Table 1. The results show that the speed of movement appears to be characteristic for each strain, and is less in the case of two of the variants (K-12–1 and W-677–7) than in the corresponding parent strains, but in one strain, 58–161/1, it is almost identical with the speed of the parent strain.

Table 1. *Movement of* Escherichia coli *K*-12, *its auxotrophs, and small-colony variants along a* 1 *mm. capillary of* 0·4 % *nutrient agar after incubation at* 37° *for* 18 *hr.*

Strain ...	K-12	58–161	W-677	K-12–1	58–161/1	W-677–7
Movement (cm.)	29	32	24	17	32	15

In liquid medium (either nutrient broth or minimal medium) the rates of multiplication of the small-colony variants were always less than the rates of multiplication of the corresponding normal strains. For example, in nutrient broth at 37° the growth rate of *Escherichia coli* K-12 from an inoculum of *c.* 100 cells was characterized by a mean generation time of 20 min. between the densities of 10^4 and 10^3 cells/ml., whereas its small-colony variant K-12–1 had a mean generation time of 25 min. There did not appear to be any difference in the lag phases, such as occurred with the small-colony variants of *Staphylococcus aureus* isolated by Swingle (1935). We must conclude therefore that the reason for the difference in colonial sizes on solid media is a slower rate of multiplication of the small-colony variants.

Recombination experiments

The results of mating the small-colony variants by the standard techniques (Clowes & Rowley, 1954*a*) with normal-sized colony parents of opposite mating type are shown in Table 2; Table 3 shows the corresponding results obtained from matings of normal-sized colonial parents. In the case of W-677–7/St^R/F – (the small-colony variant of W-677/St^R/F –), when mated with K-12 F+ on MM medium + streptomycin (Table 2, line 1), both the recombination rate and transfer of sugar fermentation markers paralleled the corresponding cross between two normal-sized colony parents (Table 3, line 1). The percentage transfer of the colonial character of the F+ parent, in this instance normal size, was 1%. This implied that it is unlikely that the small-colony character of W-677–7 is controlled by cytoplasmic genetic units, because otherwise it would be expected that the colony size of the progeny would be either mostly normal or all small, depending whether cytoplasmic fusion is or is not a necessary preliminary to recombination. The independence of cytoplasmic control is supported by the results of a similar cross where the variant was mated to a norleucine-resistant mutant of *Escherichia coli* K-12 on MM medium + norleucine (Nl) + threonine (T) + leucine (L) + thiamine (B_1) (Table 2, line 2). In this cross, by transferring a different part of the F+ chromosome

Table 2. *Matings of strains of* Escherichia coli, *one parent being a small-colony variant*

Recombination rate is expressed as: $\dfrac{\text{no. prototrophs/plate}}{\text{no. F}-\text{ cells/plate}}$.

	Mating					Percentage transfer of markers from F+ parent						
						Sugar fermentation on EMB						
No.	F+ parent	F− parent	Medium of selection	Recombination rate	No. of recombinations tested	Mal	Lac	Gal	Xyl	Mtl	Arab	Col. size
1	K-12	× W-677–7/St^R	MM + St	*c.* 10^{-6}	83	2	25	1	4	5	2	1
2	K-12/Nl^R	× W-677–7/St^R	MM + St + Nl + T + L + B_1	*c.* 10^{-8}	211	13	51	97	18	21	76	35
3	58–161	× W-677–7/St^R	MM	*c.* 10^{-6}	98	12	31	8	7	7	10	8
4	W-677	× 58–161/1/St^R	MM	*c.* 10^{-8}	320	61	15	26	58	55	23	52
5	58–161/1/St^R	× W-677	MM	*c.* 10^{-6}	147	2	24	3	4	6	3	4
6	K-12–2	× W-677/St^R	MM + St	*c.* 10^{-6}	248	5	39	3	11	11	2	1
7	K-12–2/Nl^R	× W-677/St^R	MM + St + Nl + T + L + B_1	*c.* 10^{-8}	100	6	58	96	14	18	66	0
8	K-12–2	× 58–161/St^R	MM + St	*c.* 10^{-6}	184	—	—	—	—	—	—	1
9	K-12–2	× W-677/St^R	MM + Mal + T + L + B_1	*c.* 10^{-7}	163	100	26	2	86	84	1	1

MM = minimal medium, St = streptomycin, Nl = norleucine, T = threonine, L = leucine, B_1 = thiamine, Lac = lactose, Mal = maltose, Gal = galactose, Xyl = xylose, Mtl = mannitol, Arab = arabinose.

(that containing the Nl^R locus instead of the loci T^+, L^+ and B_1^+), not only is the transfer of sugar markers changed (and resembles the pattern from the corresponding normal mating shown in Table 3, line 2), but the percentage of progeny resembling the F+ parent in colonial size is increased from 1 to 35 %. If by selecting a different part of the F+ chromosome we alter the percentage transfer of a character, we may infer that the character is determined by a locus on the chromosome; this eliminates the possibility of cytoplasmic control. Moreover, this locus which controls small-colony size is seen to be located in the region of the Nl locus. In Table 2, line 3, the mating of this variant with a methionine-requiring K-12 *E. coli* auxotroph, 58–161/F+, is summarized. The transfer of sugar markers resembles the parallel normal-colony cross (Table 3, line 3) and 8 % of the progeny resemble the F+ parent in having normal colonial size. Although in this cross we are also selecting the loci T^+, L^+ and B_1^+ from the F+ parent (as in Table 2, line 1) the 'contra-selected' marker (Clowes & Rowley, 1954*b*) in this instance is M^- instead of St^S. That the percentage transfer of colonial size is increased suggests that the locus controlling this character is more closely linked to St than to M, and from the mating shown in Table 2, line 2, lies even closer to the Nl locus.

Table 3. *Matings of strains of* Escherichia coli *K*-12, *both parents being of normal-colony size*

No.	Mating		Medium of selection	Recombination rate	No. of recombinations tested	Percentage transfer of sugar markers of F+ parent, scored on EMB.					
	F+ parent	F− parent				Mal	Lac	Gal	Xyl	Mtl	Arab
1	K-12	× W-677/St^R	MM+St	*c.* 10^{-6}	226	4	26	1	13	12	0
2	K-12/Nl^R	× W-677/St^R	MM+St+Nl+ T+L+B_1	*c.* 10^{-8}	184	15	38	93	27	26	73
3	58–161	× W-677/St^R	MM	*c.* 10^{-6}	240	6	27	4	12	12	5
4	W-677	× 58–161/St^R	MM	*c.* 10^{-6}	412	21	4	8	26	25	7
5	K-12	× 58–161/St^R	MM+St	*c.* 10^{-6}	—	—	—	—	—	—	—

Matings involving other small-colony variants do not lend themselves to such a simple genetic interpretation. In Table 2, line 4, results of the mating of 58–161/1/St^R/F−, a small-colony variant of 58–161/St^R/F−, with W-677/F+ on MM medium is shown. Here not only is the recombination rate only 2 % of that found in the parallel large colony cross (Table 3, line 4), but among the progeny there is a much larger percentage transfer of sugar markers. Consequently, the appearance of 52 % large colonies among the recombinants cannot be interpreted as being due to a simple transfer of a gene linked to the M^+ locus. When 58–161/1 was made F+ it recombined normally with W-677/F− (Table 2, line 5), the recombination rate and percentage transfers of unselected markers paralleling the normal 58–161/F+ × W-677 F− cross (Table 3, line 3). Since 4 % of the progeny were of small colonial size, this again suggests that the variant did not arise as a result of cytoplasmic variation. The other small-colony variant of 58–161, 158–161/2 behaved like 58–161/1. It was therefore thought to be the same strain, and the results of its matings are pooled with those of 58–161/1.

The small-colony variants of *Escherichia coli* K-12, namely K-12–1 and K-12–2, were mated with various auxotrophs. Both strains behaved similarly with regard to the recombination rate, and in the percentage transfer of unselected markers, and were thus assumed to be identical, the results being accordingly pooled. The matings of K-12–2 (or K-12–1) are shown in Table 2, lines 6–9. Its recombination rate and sugar transfer were normal (cf. Table 3, lines 1, 2 and 5), but whether the loci, T^+, L^+, B_1^+ or Nl^R or M^+ were selected there was little or no transfer of the small colony character. This might be due to the linkage of the locus which controls the small colony character with the locus St^S, which must always be selected against because of the prototrophy of this variant. An attempt was made to resolve this possibility by selecting the recombinants from a cross of K-12–2 with W-677/St^R/F− on MM medium + maltose in place of glucose + threonine + leucine + thiamine. By this method selection is made for the locus Mal^+ from the F+ parent which is known to be located very near to the St locus. However, as the results show in Table 2, line 9, this was not successful in bringing over the small colony character of the F+ parent.

The results shown in Table 2 suggest that the small-colony character is controlled by mutation of one of several nuclear genes, and that the mutations in each strain are non-allelic. This was verified by inter-crossing the small-colony variants. Table 4 shows that, under these circumstances, it is possible to obtain progeny of large-colony size by mating any two of the small-colony variants under the appropriate conditions of selection.

Table 4. *Intercrosses of small-colony variants of* Escherichia coli *K*-12 *and its auxotrophs*

	Mating				
No.	F+ parent	F− parent	Medium of selection	Selected markers from F+ parent	Percentage progeny of normal colonial size
1	K-12–2	× W-677/7/7St^R	MM + St	$T^+L^+B^+_1$	0
2	K-12–2/Nl^R	× W-677/7/St^R	MM + St + Nl + T + L + B_1	Nl^R	28
3	K-12–2	× 58–161/1/St^R	MM + St	M^+	15
4	W-677/7/St^R	× 58–161/1/St^R	MM	M^+	38
5	58–161/1/St^R	× W-677/7/St^R	MM	T + L + B_1	5

The speeds of movement of recombinants from the mating of K-12/Nl^R/F+ (which has a relatively high speed of movement and has a relatively large colony size) with W-677–7/St^R/F− (which has a slower speed of movement and a smaller colony size) which appeared on MM medium + St + Nl + T + L + B_1 were determined by the capillary method. In this mating, where the F+ parent has the characters high speed and large colony and the F− parent slow speed and small colony, it is seen from Table 2, line 2, that some recombinants are small and some are large, owing to the transfer of the locus for large-colony size from the F+ parent to the F− parent. However, when large and small recombinants were examined, both fast and slow speeds of movement were found in each class.

DISCUSSION

A fundamental difficulty in attempting to identify the origin of small-colony variants has been the great diversity in the properties of the variants isolated. Lindegren (1935) suggested that the methods of isolation and properties of small-colony variants isolated by certain workers could be explained by a 'transgenation', a mutation at a circumscribed locus on the genetic material; this view was supported by Braun (1947). From the present study, the resemblance of the small-colony variants isolated from *Escherichia coli* K-12 and its auxotrophs to the original normal-sized colony strains in all characters, other than rate of multiplication, suggests that they may be of the kind postulated by Lindegren, and have arisen as a result of the mutation of a single nuclear gene. In particular the genetic studies with the variant W-677–7 seem to support this conclusion.

The recombination results of the other variants (58–161/1 and K-12–1) do not allow such simple inferences. In recombinations with 58–161/1 as the F − parent, the rate of recombination is lower and the transfer of F + markers to the recombinants is greater than in corresponding crosses with the normal-sized colony parent. This suggests that additional loci from the F + parent must be transferred to yield prototrophic recombinants, and points to a growth requirement for the small-colony variant in addition to that required by the parent strain. However, since strain 58–161/1 will grow on MM medium supplemented with methionine, the zygote derived from the 58–161/1 cell would be expected to grow on MM medium, albeit more slowly, when only the M^+ locus was transferred from the F + parent, and this would give a predominance of small-colony progeny. It appears therefore that there is some interference with the ability of 58–161/1 to fulfil the role of gene acceptor.

It has been shown by matings involving K-12–2 that selection for the genetic loci investigated, which cover all the well-studied parts of the K-12 chromosome, does not result in transfer of the small-colony character to any significant degree. Therefore it must be concluded that if this character is controlled by a nuclear genetic locus, either the locus is very strongly linked with the St locus, which is necessarily a 'contra-selected' F + marker in these matings, or it lies on some part of the chromosome that is not transferred, either because of its remoteness from the selected markers or its proximity to a possible constant point of elimination. Nuclear control has, however, not been clearly demonstrated in this instance because of our inability to vary the amount of transfer of this small-colony character by selecting loci in different regions of the chromosome.

It seems likely from the segregation of colonial size and speed of movement found in the recombinants of certain matings, that these two characters are not controlled by the same gene. However, the results do not allow differentiation of cytoplasmic or nuclear control of speeds of movement. Differences in speeds were reported by Ogiuti (1936), who found from direct observation of the microscope field that whereas all strains of most motile species had the same speeds, in *Escherichia coli* there was a twofold inter-strain variation.

Of two of our strains examined under the microscope, one strain, characterized as a fast swimmer by the capillary method, was well flagellated and had a high population of motile cells, whereas the other, characterized as a slow swimmer, had fewer motile individuals and a smaller number of flagella/cell. The flagellar wavelength in the two strains was 2·53 and 2·51 μ respectively (Dr E. Leifson, personal communication).

The effect of $CuSO_4$ on *Escherichia coli* in the production of small-colony variants was interpreted as an inductive process by Weed & Longfellow (1954) who found that a population of *c.* 150 cells of *E. coli* B gave rise to small-colony variants in an inhibitory concentration of copper. It was suggested that simple selection of spontaneous mutants would not explain this phenomenon, although the small-colony variants were more resistant to the concentration of copper used in the isolation than was the original strain. The inductive effect of copper was compared with the effect of acriflavine on yeast which Ephrussi (1953) ascribed to an interference with reproduction of cytoplasmic genetic units. However, in the isolation of the small-colony variants described in the present paper, these variants were isolated from cultures at the threshold of inhibition and the variants so isolated did not have any greater resistance to the bactericidal action of copper. In most instances the conditions of isolation do not exclude their origin by spontaneous mutation at a rate of about 10^{-5}/cell division.

The slower rate of cell-division in liquid medium and on most solid media, and in particular on a partially defined medium as in MM medium, suggests that the variant does not differ by the requirement for a growth factor but by some non-specific factor such as a variation in the permeability of the cell wall to most nutrients. However, the ability to vary the size ratios illustrated by the larger size of the small-colony variants on veal digest + glucose agar and the smaller size of the original strains on PLYP agar (both compared with nutrient agar) might indicate that some growth factor, as yet unidentified, is present in suboptimal amounts in these media.

Many of the properties of small-colony variants can be accounted for by an overall lowering in the metabolic rate, as suggested by Youmans (1937). This explanation might account for the isolation of many of these variants from toxic environments, in which the inhibitor permeated the small-colony variants at a lower rate and thus permitted their survival for longer than the corresponding, normally metabolizing organisms. This general lowering of the metabolic rate might conceivably be due to a decrease in cell wall permeability.

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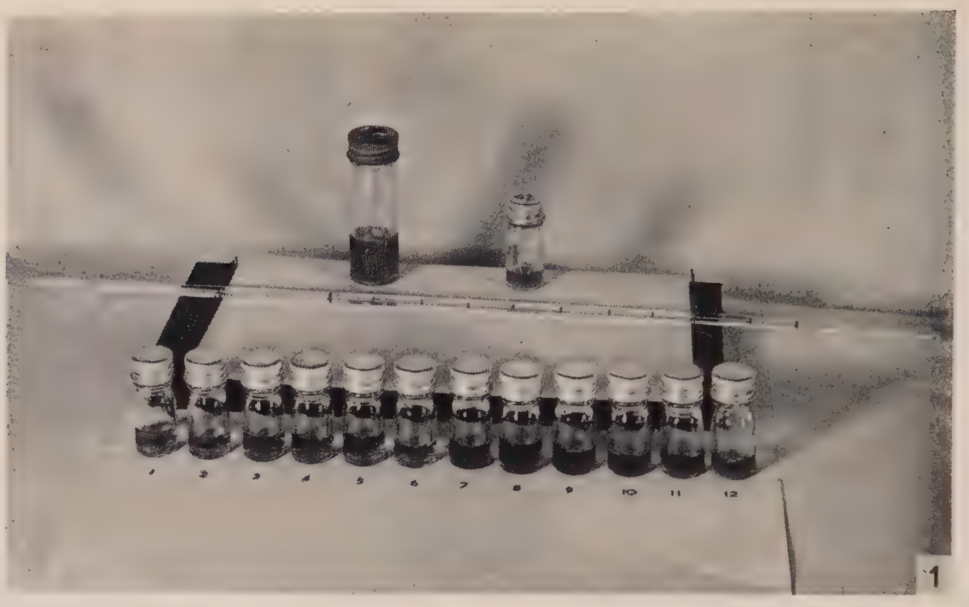
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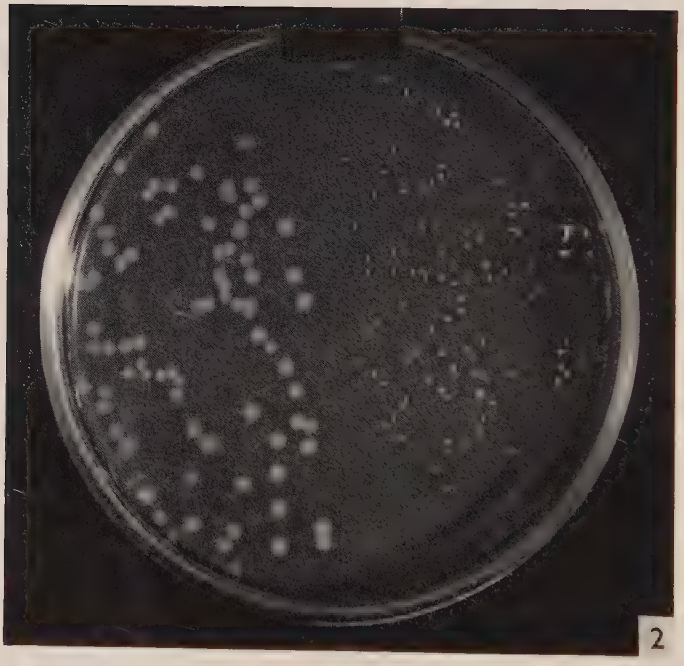

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EXPLANATION OF PLATE

Fig. 1. Apparatus used in the determination of speeds of motility.

Fig. 2. Growth of *Escherichia coli* K-12 and small colonial variant on nutrient agar for 18 hr. at 37°. (Left-hand side parent K-12; right-hand side variant K-12–1).

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The Minimal Nutritional Requirements of Some Species in the Genus *Bacillus*

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SUMMARY: The characteristic nutritional patterns were determined for seven species or varieties of the genus *Bacillus*. Six strains of *Bacillus cereus* var. *anthracis* grew in a complex but defined amino acid medium + thiamine. Three strains of the insect pathogen *B. cereus* var. *thuringiensis* had the same nutritional pattern as the parent species *B. cereus* and grew in a seven amino acid medium. Of twenty strains of *B. firmus*, ten grew on the seven amino acid medium + biotin while the growth of five other strains was markedly affected by the addition of thiamine. Two of the remaining five strains required thiamine on first isolation, but soon dispensed with it on subculture, while the other three strains continued to require thiamine. Six strains of *B. lentus* grew in the seven amino acid medium + biotin + thiamine + urea. When the seven amino acid medium was replaced by the complex amino acid medium three of these strains of *B. lentus* grew without the addition of urea, and the remaining three strains required either urea or ammonium chloride. The sixteen strains of *B. coagulans* all grew in the complex amino acid medium + biotin + thiamine. The three strains of *B. pulvifaciens* grew in the seven amino acid medium + biotin.

The twenty-seven strains of *Bacillus circulans* formed a nutritionally heterogeneous group. Eight strains were maintained in serial subculture in broth but could not be grown in defined media. One strain grew in the ammonia basal medium alone, others required added biotin or added biotin + thiamine and others required amino acid media + biotin + thiamine.

In our previous survey of the nutritional requirements of mesophilic species in the genus *Bacillus* (Knight & Proom, 1950) a number of species was either not examined or difficulties were encountered in determining their exact nutritional requirements. This supplementary paper fills some of the gaps left in our previous work.

METHODS

Strains. On isolation, or receipt from other collections, the strains were freeze-dried and deposited in the culture collection at the Wellcome Research Laboratories; in the text the reference CN refers to the Catalogue Number in this collection. Where the strain was not isolated by us the reference number of the sender is given in brackets. The strains after drying were tested for purity and identity by the methods described by Smith, Gordon & Clark (1952) and by Knight & Proom (1950).

Nutritional requirements. The methods used were similar to those described by Knight & Proom (1950). For a detailed description reference should be made to that publication, but for convenience the salient points are here given.

The usual precautions were taken to exclude contamination by unwanted nutrients. The starting-point of an experiment was a freeze-dried culture from the collection. At least five serial subcultures were made in defined media to exclude the possibility of carry-over of essential nutrients. At the conclusion of each experiment the final culture was checked for purity and identity. To exclude the possibility of selection of less-exacting variants the original strain from the collection was tested directly for its ability to grow on the medium finally selected as satisfactory for the given organism.

The media were dispensed in 5 ml. lots in 6 × 1 in. rimless glass tubes closed by loosely fitting aluminium caps. The cultures were incubated at the required temperature in a sloped position to give good aeration. The initial inoculum was taken from an agar slope with a small loop; subsequent inoculations were made with a small loop.

MEDIA

Ammonia basal salt medium. KH_2PO_4, 1·5 g.; $(NH_4)_2HPO_4$, 7·0 g.; $MgSO_4.7H_2O$, 0·5 g.; $CaCl_2.2H_2O$, 0·3 g.; $MnSO_4.4H_2O$, 0·04 g.; $FeSO_4.7H_2O$, 0·025 g.; ammonium molybdate, 0·002 g. These compounds were dissolved in distilled water to 1 l. and the pH value adjusted to 7·6 with NaOH. The solution was boiled, filtered through paper and sterilized for 20 min. at 115°.

Seven amino acid medium. Ammonia basal salt medium supplemented with the following amino acids in the concentrations shown (mg./ml.): L-asparagine, 0·4; L-proline, 0·1; L-leucine, 0·57; DL-alanine, 0·38; L-glutamic acid, 1·4; DL-serine, 0·12; DL-methionine, 0·06. The amino acid mixture was prepared separately by solution in distilled water at 10 times these concentrations. It was filtered through paper and sterilized by Seitz filtration. It was then added aseptically to the ammonia basal salt medium to give the required concentration of amino acids.

Complex amino acid medium. Ammonia basal salt medium supplemented with the following amino acids in the concentrations shown (mg./ml.): DL-alanine, 0·38; DL-aspartic acid, 0·89; L-arginine HCl, 0·3; L-cystine, 0·02; L-glutamic acid, 1·4; glycine, 0·17; L-histidine HCl, 0·24; DL-isoleucine, 0·76; L-leucine, 0·57; L-lysine HCl, 0·24; DL-methionine, 0·06; L-proline, 0·11; DL-serine, 0·12; DL-threonine, 0·10; L-tyrosine, 0·06; DL-valine, 0·15. The amino acid mixture was prepared separately by solution in distilled water at 10 times the required concentration, filtered through paper and sterilized by Seitz filtration. It was then added aseptically to the ammonia basal medium to give the required concentration.

RESULTS

The minimal nutritional requirements of collections of strains of the following seven species or varieties were examined: *Bacillus cereus* var. *anthracis*, *B. cereus* var. *thuringiensis*, *B. firmus*, *B. lentus*, *B. coagulans*, *B. pulvifaciens* and *B. circulans*. The results are summarized in Table 1 and the detailed results with each species are given below.

Bacillus cereus var. *anthracis*

Six strains of *Bacillus cereus* var. *anthracis* were examined: CN 1874 ($34F_2$) avirulent strain from Ministry of Agriculture Laboratories, Weybridge; CN 3472 ($34F_2$); CN 3482 (17JB); CN 3518 (V8R) (these three strains from Dr M. Sterne); CN 2207 (ATCC 9660); CN 1995, a freshly isolated virulent strain from a pig. All six strains grew in the complex amino acid medium supplemented with thiamine. The requirement for thiamine was absolute in that no growth took place without it and no other vitamin had to be added. The efficacy of possible precursors of thiamine was not examined. Adenosine or adenylic acid sometimes improved growth, but neither was essential. Strains usually grew for one or two subcultures in the complex amino acid medium without added thiamine but they could not be maintained in serial subculture. The amino acid requirements were complex; the strains would grow on the seven amino acid medium + thiamine for a few subcultures but soon died out. They would not grow at all on the seven amino acid medium without thiamine.

Table 1. *The minimal nutritional requirements of some species in the genus* Bacillus

Species	No. of strains examined	Nutrition
B. cereus var. *anthracis*	6	Complex NH_2 + Th.
B. cereus var. *thuringiensis*	3	$7NH_2$
B. firmus	10	$7NH_2$ + Bio.
	5	$7NH_2$ + Bio.; Th. improves growth.
	2	$7NH_2$ + Bio. + Th.; but in subculture can soon dispense with Th.
	1	$7NH_2$ + Bio. + Th.
	2	Complex NH_2 + Bio. + Th.
B. lentus	3	Complex NH_2 + (urea *or* NH_4) + Bio. + Th. or $7NH_2$ + urea (not NH_4) + Bio. + Th.
	3	Complex NH_2 + Bio. + Th. or $7NH_2$ + urea + Bio. + Th.
B. coagulans	16	Complex NH_2 + Bio. + Th.
B. pulvifaciens	3	$7NH_2$ + Bio.
B. circulans	1	NH_4
	3	NH_4 + Bio.
	6	NH_4 + Bio. + Th.
	3	$7NH_2$ + Bio. + Th.
	6	Complex NH_2 + Bio. + Th.
	8	Complex undefined media (e.g. nutrient broth)

Complex NH_2 = complex amino acid medium; $7NH_2$ = seven amino acid medium; Bio. = biotin; Th. = thiamine.

Bacillus cereus var. *thuringiensis*

Three strains of this insect pathogen were received from Dr W. C. Haynes: CN 3624 (NRS 996); CN 3625 (NRS 1124); CN 3626 (NRS 1328). All strains grew in the seven amino acid medium, the requirement for amino acids was absolute and no other growth factors were required.

Bacillus firmus

Twenty strains of *Bacillus firmus* were examined; of these strains fifteen were received from Dr Ruth Gordon, one from the American Type Culture Collection (ATCC) and one from the National Collection of Type Cultures (NCTC) and three were fresh isolates from soil. Ten of these strains—CN 2196 (ATCC 8247); CN 2544; CN 3316 (854); CN 3317 (858); CN 3318 (861); CN 3319 (855); CN 3384 (1070); CN 3386 (1122); CN 3390 (1151); CN 3389 (1149)—grew in the seven amino acid medium + biotin; the requirements for amino acids and biotin were absolute. Five other strains—CN 2936; CN 1758 (NCTC 6354); CN 3315 (783); CN 3314 (613); CN 3320 (860)—grew indifferently in the above medium, and the growth was markedly improved by the addition of thiamine. Two other strains—CN 3385 (1131); CN 3392 (1329)—required the addition of thiamine, but on serial subculture in defined medium variants were readily selected which grew in the absence of thiamine. Three other strains—CN 1654; CN 3391 (1152); CN 3387 (1147)—showed an absolute requirement for thiamine. Two of the strains—CN 3391; CN 3387—had more complex amino acid requirements and would only grow with the complex amino acid medium + biotin + thiamine.

Bacillus lentus

Six strains of *Bacillus lentus* were examined: two strains—CN 2788; CN 2789—were fresh isolates from soil; three strains—CN 3323 (670); CN 3321 (769); CN 3323 (749)—were received from Dr Ruth Gordon; and one strain—CN 2750 (165)—from Dr T. Gibson. All strains showed an absolute requirement for amino acids + thiamine + biotin. With the complex amino acid medium three strains—CN 2750; CN 2789; CN 3323—required also the addition of urea or ammonium chloride. The growth with urea was uniformly better than with ammonium chloride. All six strains required urea in the presence of the seven amino acid medium and in this medium urea could not be replaced by ammonium chloride.

Bacillus coagulans

Sixteen strains of *Bacillus coagulans* were examined; one strain was from the American Type Culture Collection, ATCC 7050, and the remaining strains were fresh isolates from soil. These strains formed a nutritionally homogeneous group and all grew in the complex amino acid medium + biotin + thiamine. The amino-acid requirements were complex and the complex amino acid medium could not be replaced by the seven amino acid medium.

Bacillus pulvifaciens

Three strains of *Bacillus pulvifaciens*—CN 3621 (NRS 1283); CN 3622 (NRS 1284); CN 3623 (NRS 1285)—were received from Dr W. C. Haynes. All three strains grew in the seven amino acid medium + biotin. The requirements for amino acids and biotin were absolute and no other growth factors were required.

Bacillus circulans

Twenty-seven strains of *Bacillus circulans* were examined; of these strains sixteen were fresh isolates from soil, nine were from Dr T. Gibson and one strain each was received from the American Type Culture Collection and from the National Collection of Type Cultures. In contrast to all the other collections of cultures representative of species within the genus *Bacillus* which we classified according to the criteria of Smith *et al.* (1952), these twenty-seven strains of *B. circulans* formed a nutritionally heterogeneous group. One strain, CN 2719, grew in the ammonia basal medium alone; added amino acids or growth factors were not required. Three strains—CN 2718; CN 2923; CN 3377 (287)—grew when biotin was added to the ammonia basal medium. Six strains—CN 2720; CN 2721; CN 2848; CN 2905; CN 2925; CN 3378 (1375)—grew when biotin and thiamine were added to the ammonia basal medium. Three strains—CN 2924; CN 2201 (ATCC 1649); CN 3375 (109)—grew in the seven amino acid medium + biotin + thiamine. Six strains—CN 2849; CN 2621; CN 2723; CN 2722; CN 2526; CN 3371 (558)—had more complex amino acid requirements and grew in the complex amino-acid medium + biotin + thiamine. The remaining eight strains—CN 2628; CN 2629; CN 1609 (NCTC 6351); CN 3370 (560); CN 3733 (261); CN 3381 (796); CN 3374 (613); CN 3379 (110)—were still more exacting in their nutrient requirements. These eight strains could be maintained in serial subculture in meat broth or in nutrient broth, they could also be maintained with some difficulty in peptone water but we were unable to grow them in defined media.

DISCUSSION

This supplementary survey of the nutrition of strains of certain species of *Bacillus* shows, with one exception to be discussed later, the same uniformity of nutritional patterns within species that was found in our previous work (Knight & Proom, 1950). The six strains of *B. anthracis*, including avirulent and virulent strains, all grew on a complex mixture of amino acids + thiamine; the addition of adenosine or adenylic acid sometimes improved growth but neither was essential. These results confirm the observation of Brewer *et al.* (1946) with the Vollum M 36 strain. The nutritional pattern of *B. anthracis* was therefore different from that of the parent species *B. cereus*; the *B. anthracis* strains showed a nutritional requirement satisfied by thiamine and they were more exacting in their amino acid requirements. This nutritional difference might be used as evidence to support the classification of *B. anthracis* as a separate species rather than as a variety of *B. cereus*.

In contradistinction to the animal pathogen *Bacillus anthracis*, the insect pathogen *B. thuringiensis* had the same nutritional pattern as the parent species *B. cereus* and showed a requirement for amino acids only.

The twenty strains of *Bacillus firmus* all showed a nutritional requirement satisfied by amino acids + biotin, but they differed amongst themselves in their requirements for thiamine. According to the strain the addition of thiamine either made little difference, improved growth or was essential. The nutritionally most exacting strain of *B. firmus* had the nutritional pattern of the least exacting strain of *B. lentus*.

The *Bacillus firmus* and *B. lentus* strains may be arranged in an order which suggests a nutritional series with the least exacting strains, requiring a simple mixture of amino acids + biotin and the most exacting a complex mixture of amino acids + urea + biotin + thiamine. In addition, the *B. lentus* strains showed a requirement satisfied by urea or by ammonia, according to the mixture of amino acids present. This gradation in nutritional pattern without the usual clear-cut differences is reflected in the difficulty in finding adequate bacteriological tests for distinguishing *B. firmus* from *B. lentus*. It is also in keeping with the observation made previously (Knight & Proom, 1950) that a disproportionate number of intermediates was encountered when strains of these species were isolated from soil: of eleven isolates clearly belonging to the firmus-lentus group, only five could be allocated to one or other species and six were intermediate.

The sixteen strains of *Bacillus coagulans* examined formed a nutritionally homogeneous group and all grew on the complex amino acid medium + biotin + thiamine. No other growth factors were required, and without biotin and thiamine there was no growth. Some strains occasionally showed growth for a few subcultures without added biotin or thiamine but for serial subculture both substances were needed. These results differ from those reported by Cleverdon, Pelczar & Doetsch (1949) and by Allen (1953); we have no explanation for the differences.

In previous work (Knight & Proom, 1950) we found difficulty in determining the nutritional requirements of *Bacillus firmus* and *B. lentus* isolates, and their nutrition was described as complex. Also we were unable to replace the hydrolysed casein by a defined amino acid mixture for *B. coagulans* isolates. These effects were not due to a missing growth factor but, it now appears, to the presence of inhibitors. Proom (1955) showed that growth inhibitory concentrations of colloidal sulphur or colloidal copper sulphide may be formed during preparation of defined media containing mixtures of amino acids. These inhibitors were responsible, for example, for the failure of cultures of *Bordetella pertussis* to grow in a defined amino acid medium, whereas the same strains grew in the presence of hydrolysed casein. The method of preparation of amino acid mixtures used in our previous work did not exclude the possibility of the production of these inhibitors. Some strains of *Bacillus firmus*, all strains of *B. lentus* and of *B. coagulans* were found to be inhibited by the complex amino acid medium which had been gassed with H_2S to remove traces of heavy metals and finally sterilized by autoclaving; but these organisms were not inhibited by the complex amino acid medium prepared in the manner described in the present paper. These species (*B. firmus*, *B. lentus*, *B. coagulans*) differ from others in the genus *Bacillus*, which are apparently not inhibited by colloidal sulphur or colloidal copper sulphide.

The nutritional requirements of the three strains of *Bacillus pulvifaciens* examined were satisfied by the seven amino acid medium + biotin. The nutritional requirements were therefore different from those of the other species in the morphological group 2 of the genus *Bacillus*.

In contrast to the strains of all other species of the genus *Bacillus* which we have examined, the 20 strains defined as *B. circulans* formed a nutritionally heterogeneous group. So far we have been unable to grow 8 of these strains in defined media, although they can be maintained in serial subculture on nutrient agar or in nutrient broth. The remaining strains grew when given respectively and depending on the strain, either ammonia, ammonia + biotin, ammonia + biotin + thiamine, seven amino acid medium + biotin + thiamine, or the complex amino acid medium + biotin + thiamine. The heterogeneity of the nutritional requirements of this group of isolates is in keeping with the physiological and biochemical variations observed within the *B. circulans* complex as at present defined and which has made classification and identification of members of this group the most difficult in the genus. If one is prepared to accept consistency of nutritional pattern as part of the characterization of species within the genus *Bacillus*, the implication is that the *B. circulans* complex is at present inadequately classified.

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The Classification of Lactobacilli from the Human Mouth

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SUMMARY: A scheme of classification for *Lactobacillus* strains from diverse sources is proposed, based upon the results obtained with 473 strains, mainly of human oral origin, using a selected range of morphological and physiological taxonomic criteria. Two subgeneric groups, containing five stable and clearly demarcated specific types are recognized.

The lack of a satisfactory classification of the genus *Lactobacillus* is reflected in the number of recent systematic studies which have been published, and which have been reviewed, together with much of the earlier work, by Briggs & Briggs (1954). Almost all the taxonomic techniques known to bacteriology have been employed, at various times, by the many workers in this field. Studies by Tilden & Svec (1952), Rogosa, Wiseman, Mitchell, Disraely & Beaman (1953), Grubb & Krasse (1953), Briggs (1953), Wheater (1955*a*, *b*) and Sharpe (1955) have clarified the status of some named species, but, as will become evident, it is not considered that any of these, or indeed the present work, provides the final word upon the subject.

The object of this investigation was to examine and select a combination of simple and easily applied physiological tests, in addition to the morphological criteria already described (Davis, Bisset & Hale, 1955), whereby the oral lactobacilli might readily be identified and classified within a system applicable to the entire genus. The eventual purpose is to clarify the role of these bacteria in dental caries.

METHODS

Isolation and purification

All the strains examined were freshly isolated except 28 obtained from the National Collection of Industrial Bacteria, (NCIB), Teddington, and 27 from the National Institute for Research in Dairying, (NIRD), Shinfield, by courtesy of Dr M. E. Sharpe. Their origins, and in the case of the type cultures, their numbers and original designations, are shown in Tables 2–4.

The complex tomato juice (TJ) medium previously described (Davis *et al.* 1955) was used throughout the study for routine cultivation of *Lactobacillus*. It is based upon those of Kulp (1927), Rogosa, Mitchell & Wiseman (1951) and Briggs (1953), and gave satisfactory results both in liquid form and when solidified by the addition of 2·5% agar.

Primary cultures were made in broth in bijou bottles, and the growth was plated out at intervals over a period of 4–6 days of incubation at 37°. Colonies were picked from 48 hr. cultures on the solid medium, and the strains were purified by alternate growth in broth for 24–96 hr., from which colonies were again picked. The process was repeated until uniformity of appearance in agar surface cultures indicated purity.

Although good growth was obtainable in 24 hr. in TJ broth, prolonged incubation, for 4 to 6 days, greatly assisted the isolation of lactobacilli, because they survived the conditions of falling pH value much more easily than did most of the other oral bacteria, e.g. streptococci.

Table 1. *Comparison of overall group characteristics*

Character ...	Homofermentative			Heterofermentative	
	Group 1	Group 2	Group 3	Group 4	Group 5
Colony ...	Med/FRI	MS/SR	S	S/SR	RI
Cell type ...	Long bacilli	Curling chains	Short single and paired bacilli	Short single and paired bacilli	Short to medium bacilli
Growth at 15°	−	+	+	−/W	−
Growth at 45°	+	+/W	−/W	+	+
Arginine	−	−	−	+	$-\left(\frac{19}{23}\right)$
Aesculin	+/−	+	+	−	+
Hippurate	−/W	+	+	−	+
Acetoin	−/W	W/+	+	−	−
Raffinose	±	−	±	±	±
Mannitol	−	+	+	−	−
Sorbitol	−	±	+	−	−
Salicin	±	+	+	−	±
Rhamnose	−	±	+	−	−
Lactose	+	±	+	±	+
Melibiose	±	∓	±	±	+
Xylose	−	−	∓	∓	±
Arabinose	−	−	±	∓	+
Mannose	+	+	+	∓	−
Melezitose	−	±	∓	−	−
10 % Bile	±	±	+	+	−
0·3 % Phenol	+	+	+	+	+
0·4 % Phenol	±	±	+	+	+
4 % NaCl	±	+	+	+	+
8 % NaCl	−	±	+	−	−
Acid from glucose	1–6 ml.	4–6 ml.	4–6 ml.	2–4 ml.	1–3 ml.

Colonies: Med = 'Medusa-head'; F = flat; R = rough; S = smooth; I = irregular; M = 'Mucoid'.

+ = normally positive; − = normally negative; ± = over 50 % of strains positive; ∓ = less than 50 % of strains positive; W = weak reaction.

When TJ medium was used, no special techniques for the production of anaerobic or microaerophilic conditions were necessary. All strains of lactobacilli encountered grew well at 37° under aerobic conditions. When not required for immediate examination, strains were maintained in cooked meat medium (Mackie & McCartney, 1950, p. 185), as recommended by Sullivan (1939).

Preliminary identification

The strains to be examined were first identified as members of the genus *Lactobacillus*. Gram-staining, general morphology and motility were examined in 24 hr. TJ broth cultures.

Catalase production was tested by pipetting one drop of 3% (v/v) hydrogen peroxide over 48 hr. discrete surface colonies, and examining for effervescence under the wide-field microscope. Candidas and most staphylococci were readily detected by this means.

Table 2. *Classification of NCIB strains*

Present grouping	NCIB no.	Deposited as
1	8118	*L. leichmanii*
	8170	*L. lactis*
	4504	*L. acidophilus*
	1723	*L. acidophilus*
2	1407	*L. acidophilus-odontolyticus*
	1406	*L. acidophilus-odontolyticus*
	8103	*L. helveticus*
	7473	*L. delbrueckii*
	8019	*L. casei*
	8010	*L. casei*
	6375	*L. helveticus*
	8125	*L. delbrueckii*
3	6461	*L. plantarum*
	4504a	*L. acidophilus*
	8531	*L. plantarum*
	8026	*L. plantarum*
	4505	*L. acidophilus*
4	8028	*L. fermenti*
	6991	*L. fermenti*
	7230	*L. fermenti*
5	8007	*L. buchneri*
	947	*L. brevis*
	8038	*L. brevis*
Not lactobacilli	8130	*L. delbrueckii*
	8121	*L. enzymothermophilus*

Nitrate reduction was tested in the following medium: Oxoid peptone, 0·5% (w/v); Difco yeast extract, 0·3% (w/v); glucose, 0·5% (w/v); agar, 0·1% (w/v); potassium nitrate (KNO_3), 0·2% (w/v); Salts A, 0·5% (v/v); and Salts B, 0·5% (v/v). The pH was adjusted to 6·8 and the medium dispensed into $5 \times \frac{1}{2}$ in. tubes, each half-filled, and autoclaved at 15 lb. for 15 min. The inoculum consisted of 2 or 3 drops of 24 hr. TJ broth culture delivered from a capillary pipette. The tests were incubated for 6 days at 37°, and to each test was added one drop of dilute iodine solution and 1 ml. of each of the Griess-Ilosvay reagents (Topley & Wilson, 1946, p. 368). The tests were allowed to stand at room temperature for 30 min. and the results noted; false negative reactions were checked by the addition of powdered zinc and further observations of colour change. The presence of adequate growth was always noted before the test was made.

Polysaccharide production when grown on 5% sucrose-TJ agar was examined by observation of colonies after 4 days at 37°. The sucrose solution employed was sterilized by Seitz filtration.

Strains accepted as members of the genus *Lactobacillus* in this study were Gram-positive rods, non-sporing, non-motile; they did not produce catalase, reduce nitrate or produce copious polysaccharide material from sucrose.

Table 3. *Classification of NIRD strains* (*homofermentative only*)

Present grouping	NIRD no.	Original designation	Wheater, 1955	Sharpe, 1955
1	B2	*L. bulgaricus*	*L. bulgaricus*	*L. bulgaricus*
	B14	*L. bulgaricus*	*L. bulgaricus*	*L. bulgaricus*
	B12	*L. bulgaricus*	*L. bulgaricus*	*L. bulgaricus*
	AH4	N.C.	*L. bulgaricus-acidophilus*	N.C.
	L15	*L. lactis*	N.T.	*L. lactis*
	L1	*L. lactis*	N.T.	*L. lactis*
	L6	*L. lactis*	N.T.	*L. lactis*
	A12	*L. acidophilus*	*L. acidophilus*	N.C.
	A4	*L. acidophilus*	*L. acidophilus*	N.C.
	A1	*L. acidophilus*	*L. acidophilus*	N.C.
	A18	*L. acidophilus*	*L. acidophilus*	N.C.
	L3	*L. lactis*	N.T.	*L. lactis*
2	C5	*L. casei*	*L. casei*	*L. casei-helveticus*
	C6	*L. casei*	*L. casei*	*L. casei*
	C9	*L. casei*	*L. casei*	*L. casei*
	O7	N.C.	*L. casei*	*L. casei*
	C2	*L. casei*	*L. casei*	*L. casei-helveticus*
	C17	*L. casei*	*L. helveticus*	*L. helveticus*
	H2	*L. helveticus*	*L. helveticus*	*L. helveticus*
	H5	*L. helveticus*	*L. helveticus*	*L. helveticus*
3	P5	*L. plantarum*	*L. plantarum*	*L. plantarum*
	P4	*L. plantarum*	*L. plantarum*	*L. plantarum*
	AR1	*L. arabinosus*	*L. plantarum*	*L. plantarum*
	AR5	*L. arabinosus*	*L. plantarum*	*L. plantarum*

N.T. = not tested; N.C. = not classified.

Physiological tests

The following physiological tests were selected from the very large number recorded in the literature and modified by a process of trial and error; they were employed for the classification of *Lactobacillus* strains. All inoculations were made by sterile capillary pipette from active 24 hr. TJ broth cultures, after at least two subcultures in that medium at 24 hr. intervals. Unless otherwise indicated, the incubation temperature for all tests was 37°. The peptone used in all media was the Oxoid product and the yeast extract, Difco. The salts A and B are those employed in the TJ medium already described (Davis *et al.* 1955).

Gas production from glucose. Medium: peptone, 1% (w/v); yeast extract, 0·5% (w/v); glucose, 3% (w/v); Tween 80, 0·1% (v/v); Salts A, 0·5% (v/v); Salts B, 0·5% (v/v); pH 7·2, with 0·2% (v/v) of 1·5% aqueous solution of bromocresol-purple added. Dispensed into 5 × ½ in. tubes containing inverted Durham tubes and steamed for 20 min. on 3 consecutive days to sterilize. The casein digest fermentation test medium described below was also found to be effective as the basal medium for this test. After inoculation with 3 to 4 drops, the tests were sealed by a layer of *c.* 1 ml. of sterile liquid paraffin. They were incubated for 6 days and observed every 48 hr.

Ammonia production from arginine. Medium: peptone, 0·5% (w/v); yeast extract, 0·3% (w/v); glucose, 0·3% (w/v); sodium acetate, 1% (w/v); L-arginine hydrochloride, 0·3% (w/v); Tween 80, 0·1% (v/v); Salts A, 0·5% (v/v); Salts B, 0·5% (v/v); pH 7·4, dispensed in 4 ml. quantities in ¼ oz. (bijou) bottles and steamed 20 min. on 3 consecutive days to sterilize. Tests were inoculated with 1 to 2 drops, incubated for 10 days and tested by adding 1 ml. of Nessler's reagent to detect ammonia.

Table 4. *Source, numerical distribution and classification of* 473 *strains of* Lactobacillus

Source	Lactobacillus groups 1	2	3	4	5	Source totals
Saliva	2	33	1	75	2	113
Filled teeth	5	16	1	5	.	27
Carious teeth	19	41	.	55	8	123
Tooth surfaces	7	58	.	16	9	90
Caries—free salivas	5	32	3	10	.	50
Yoghurt	3	.	.	.	.	3
Milk	.	12	.	.	.	12
NIRD	12	11	4	.	.	27
NCIB	4	12	5	3	4	28
Group totals	57	215	14	164	23	473

Hydrolysis of sodium hippurate. Medium: peptone, 1% (w/v); yeast extract, 0·5% (w/v); glucose, 0·5% (w/v); sodium hippurate, 1% (w/v); sodium chloride, 0·5% (w/v); Tween 80, 0·1% (v/v); Salts A, 0·5% (v/v); Salts B, 0·5% (v/v); pH 6·8, bottled in *c.* 5 ml. quantities in ¼ oz. bottles and sterilized by steaming for 20 min. on 3 consecutive days.

Tests were inoculated with 1 to 2 drops, incubated for 10 days, and tested by removing two 1 ml. samples of clear supernatant culture fluid (centrifuged if necessary) to two clean 5 × ½ in. tubes. To one 0·5 ml. ferric chloride reagent (12% ferric chloride solution plus 2·5 ml. concentrated hydrochloric acid per litre) was added, and to the other 1 ml. of a 50% sulphuric acid solution. They were allowed to stand, and shaken occasionally for 30–60 min. A fine crystalline precipitate indicated a positive reaction. The role of both reagents is to detect free benzoic acid; weak positive hydrolysis however may only be detectable by the sulphuric acid test. If allowed to stand for 24 hr., large needle-like crystals of unknown composition appeared in some of the sulphuric acid tests but these did not indicate a true positive reaction.

Hydrolysis of aesculin. Medium: peptone, 1% (w/v); yeast extract, 0·5% (w/v); sodium acetate, 1%(w/v); aesculin, 0·5% (w/v); ferric ammonium citrate, 0·05% (w/v); Tween 80, 0·1% (v/v); Salts A, 0·5% (v/v); Salts B, 0·5% (v/v); at pH 6·8, dispensed in 4 ml. quantities into bijou bottles and autoclaved at 10 lb. for 20 min. to sterilize. The tests were inoculated with 1 to 2 drops, incubated for 14 days and observed at 48 hr. intervals for loss of fluorescence and blackening of the culture, indicating hydrolysis.

Acetylmethylcarbinol (acetoin) production from glucose. Medium: peptone, 0·5% (w/v); yeast extract, 0·3% (w/v); sodium acetate, 0·5% (w/v); glucose 2% (w/v); Tween 80, 0·1% (v/v); Salts A, 0·5% (v/v); Salts B, 0·5% (v/v); at pH 6·8, dispensed in 3 ml. quantities into bijou bottles and sterilized by steaming for 20 min. on 3 consecutive days.

Tests were inoculated with 1 to 2 drops, incubated for 10 days, and tested by transferring the culture to a clean test-tube, adding a few granules of creatine, 0·6 ml. of 5% α-naphthol solution in absolute ethanol and 0·2 ml. of 40% potassium hydroxide solution (Barritt, 1936). Positive reactions varied in the intensity of the red colour produced with these reagents.

Fermentation of selected carbohydrates. Medium: casein partial-hydrolysate (Peters & Snell, 1954) was prepared as follows: 112 g. of BDH light white soluble casein in 1600 ml. of N-sulphuric acid was autoclaved for 5 hr. at 15 lb. Approximately 180 g. of barium hydroxide was mixed in thoroughly; the pH re-adjusted to *c.* 3·0 with N-sulphuric acid; 56 g. of activated charcoal were added and mixed well for 30 min. The solution was filtered through paper pulp on a Buchner funnel.

A double-strength basal medium was then prepared by adding 56 g. of peptone, 28 g. of yeast extract, 5·6 ml. of Tween 80, 28 ml. of Salts A and 28 ml. of Salts B to the casein hydrolysate solution and diluting to a final volume of 2800 ml. After steaming to dissolve the solids the pH was adjusted to 7·0, the medium re-steamed for 20 min. and clarified by filtration through paper pulp. This medium was autoclaved for 15 min. at 15 lb. and stored in 250 ml. quantities. Fermentation tests were prepared by diluting 250 ml. of the double-strength medium to 450 ml., adjusting the pH to 7·2, adding 1 ml. of a 1·5% aqueous solution of bromocresol-purple, and in the case of heat tolerant carbohydrates, 50 ml. of 10% carbohydrate solution. The final medium was dispensed into bijou bottles and sterilized by steaming for 20 min. on 3 consecutive days. With those carbohydrates which are degraded by heat, the basal medium was sterilized by autoclaving, cooled, and 50 ml. of a 10% Seitz-filtered carbohydrate solution added with sterile precautions. The sterility was checked by incubation for 3 days at 37°.

The following carbohydrates were employed: D-mannose, D-xylose, L-arabinose, L-rhamnose, D-melibiose, lactose, melezitose, sorbitol, mannitol, salicin and raffinose. The first three were sterilized by Seitz-filtration.

Tests were inoculated with 1 drop, incubated for 14 days and observed at 48 hr. intervals for colour change from purple to brilliant yellow with accompanying turbidity.

Growth at 15° *and* 45°. This was tested in $5 \times \frac{1}{2}$ in. tubes of TJ broth and observed after incubation in an accurately controlled water-bath for 7 days. The inoculum was 1 to 2 drops; all strains were tested in duplicate at both temperatures.

Tolerance of inhibitory substances. TJ broth was used as the basal medium;

all tests were conducted in bijou bottles and sterilized by autoclaving at 10 lb. for 20 min. The following substances were used: 10% sodium taurocholate, 0·3 and 0·4% phenol, 4 and 8% sodium chloride. The inoculum was 1 drop. Tests were incubated for 6 days and tolerance recorded by the presence or absence of turbidity, indicating growth.

Titratable acid production from glucose. Medium: peptone, 0·5% (w/v); yeast extract, 0·3% (w/v); glucose, 0·5% (w/v); Tween 80, 0·1% (v/v); Salts B, 0·5% (v/v); at pH 7·2, accurately dispensed in 10 ml. quantities and sterilized by steaming for 20 min. on 3 consecutive days. The inoculum was 2 to 3 drops; tests were incubated for 3 days and titratable acid was recorded in terms of 0·1 N-sodium hydroxide required to neutralize to phenolphthalein. All strains were tested in duplicate and the corrected mean value recorded.

Colony form. Recorded for discrete, 48 hr. colonies on the surface of TJ agar.

Cell form. Recorded from observations made upon heat-fixed, Gram-stained films and unstained hanging-drop preparations of active 24 hr. TJ broth cultures. The results obtained by more refined methods are described elsewhere (Davis *et al.* 1955).

RESULTS

The 473 strains examined were classified into five species on the basis of the tests described (Table 1). The system embodies groupings in accordance with published definitions which appear to have the greatest validity in the light of the present findings; although the taxonomic rank allotted to them is not necessarily that used by previous workers. The results are in closest agreement with those of Rogosa *et al.* (1953), and their relationship with those of other workers is explained in the discussion.

Group 1. Lactobacillus acidophilus

(Comparable to Orla-Jensen's 1919 genus *Thermobacterium.*) 57 strains were of this type.

Species definition. No gas produced from glucose. Colony small, flat, rough or 'Medusa-head'. Cell form: always fairly long bacilli. No ammonia produced from arginine; grow at 45° but not at 15°.

Three varieties were encountered in strains of this group.

(*a*) Variety *acidophilus* (comparable to the *Lactobacillus acidophilus* of Wheater 1955*a* and Rogosa *et al.* 1953). 47 strains.

Cell form rather variable, moderate to long bacilli, frequently in rigid chains or filaments. Hydrolyses aesculin and sometimes forms acetoin; hippurate not usually hydrolysed. Ferments salicin, lactose, melibiose and mannose, and usually raffinose. Tolerates 10% bile only poorly; 0·4% phenol and 4% but not 8% sodium chloride tolerated. Acid from glucose 4–6 ml.

(*b*) Variety *bulgaricus* (comparable to the *Lactobacillus bulgaricus* of Wheater 1955*a*). 7 strains.

Bacilli long, rigid, adhering in pairs, fours and short chains. Aesculin and acetoin negative, and hippurate usually negative. Usually ferments lactose and sometimes mannose. Tolerates 0·3 % phenol but not 10 % bile, 0·4 % phenol, or 4 % sodium chloride. Acid from glucose 1–2 ml.

(*c*) Variety *lactis* (comparable to the *Lactobacillus lactis* of Sharpe 1955). 3 strains.

Cell form as for variety *bulgaricus*. Aesculin, hippurate and acetoin negative. Ferments salicin, lactose and mannose. Tolerates 0·3 %, but not 0·4 % phenol; does not tolerate 10 % bile or 4 % sodium chloride. Acid from glucose, 1–2 ml.

Group 2. Lactobacillus casei

(Included in Orla-Jensen's genus *Streptobacterium*.) 215 strains.

Species definition: no gas produced from glucose. Colony form normally large, smooth, 'mucoid' (Hadley, 1937), convex, entire. Sometimes slightly irregular and occasionally forms large, raised 'Medusa-head' colonies. Young colonies usually rough or semi-rough. Cell form: curling chains of short to medium length bacilli; bacilli tend to curve. No ammonia produced from arginine. Hippurate and aesculin always hydrolyzed. Acetoin usually formed. Grows at 15°, but poorly or not at all at 45°. Ferments mannitol, salicin, and mannose, and often sorbitol, rhamnose, lactose, melibiose and melezitose. Tolerates 0·3 % phenol and 4 % NaCl, and certain strains always tolerate 8 % NaCl (var. A below); 10 % bile and 0·4 % phenol tolerated by most strains. Acid from glucose 4–6 ml.

Five varieties are defined on the basis of fermentation patterns (Table A):

Table A. *Varieties of* Lactobacillus casei

Variety	Raffinose	Mannitol	Sorbitol	Salicin	Rhamnose	Lactose	Melibiose	Xylose	Arabinose	Mannose	Melezitose	No. of strains
A	−	+	+	$\frac{109}{111}$	+	+	$\frac{1}{111}$	−	−	+	+	111
B	−	+	+	+	−	+	$\frac{5}{78}$	−	−	+	$\frac{47}{78}$	78
C	−	+	−	+	−	$\frac{12}{13}$	$\frac{8}{13}$	−	−	+	$\frac{5}{13}$	13
D	−	+	+	+	−	−	−	$\frac{1}{10}$	$\frac{1}{10}$	+	$\frac{1}{10}$	10
E	+	+	+	+	$\frac{1}{3}$	+	$\frac{2}{3}$	−	$\frac{1}{3}$	+	+	3
											Total	215

Fractional figures indicate: $\frac{\text{no. of strains positive}}{\text{no. tested}}$.

Group 3. Lactobacillus plantarum

(Included in Orla-Jensen's genus *Streptobacterium*.) 14 strains.

Species definition. No gas produced from glucose. Colony form smooth, convex, entire. Cell form short; single and paired bacilli. No ammonia produced from arginine. Aesculin and hippurate hydrolyzed and acetoin usually formed. Grows at 15° but poorly or not at all at 45°. Ferments mannitol, sorbitol, salicin, rhamnose, lactose and mannose, often ferments raffinose, xylose, arabinose and melibiose and occasionally melezitose. Tolerates 10 % bile, 0·4 % phenol, 4 % NaCl and usually 8 % NaCl. Acid from glucose 4–6 ml.

Group 4. Lactobacillus fermenti

(Included in Orla-Jensen's genus *Betabacterium.*) 164 strains.

Species definition. Gas produced from glucose. Colony form smooth or sometimes semi-rough, convex, entire or slightly irregular, fairly large. Cell form short, single and paired, rather square bacilli. Ammonia produced from arginine. Aesculin, hippurate and acetoin normally negative. Grows well at 45° but poorly or not at all at 15°. Ferments lactose, melibiose and usually raffinose, and sometimes xylose, arabinose and mannose. Tolerates 10% bile, 0·4% phenol, 4% NaCl but not 8% NaCl. Acid from glucose 2–4 ml.

Five varieties are defined on the basis of fermentation reaction patterns (Table B).

In variety A, 14 strains hydrolyzed hippurate weakly, 3 gave positive reactions with aesculin and 16 appeared to form acetoin. Two strains of variety B hydrolyzed hippurate, and aesculin was hydrolyzed by one strain of variety D and both of variety E.

Table B. *Varieties of* Lactobacillus fermenti

Variety	Raffinose	Mannitol	Sorbitol	Salicin	Rhamnose	Lactose	Melibiose	Xylose	Arabinose	Mannose	Melezitose	No. of strains
A	+	–	–	–	–	+	$\frac{111}{114}$	$\frac{22}{114}$	$\frac{32}{114}$	$\frac{56}{114}$	$\frac{1}{114}$	114
B	–	–	–	–	–	+	$\frac{9}{16}$	$\frac{3}{16}$	$\frac{5}{16}$	$\frac{8}{16}$	–	16
C	+	–	–	–	–	–	+	$\frac{4}{17}$	$\frac{9}{17}$	$\frac{10}{17}$	–	17
D	–	–	–	–	–	–	$\frac{1}{15}$	$\frac{3}{15}$	$\frac{11}{15}$	$\frac{1}{15}$	$\frac{1}{15}$	15
E	+	–	–	+	–	+	+	–	+	$\frac{1}{2}$	–	2
											Total	164

Group 5. Lactobacillus brevis

(Included in Orla-Jensen's genus *Betabacterium.*) 23 strains.

Species definition. Gas produced from glucose. Colony form small, raised, 'Medusa-head', or rough, or semi-rough. Cell form short to medium length bacilli, in pairs and sometimes short chains. Ammonia not usually produced from arginine. Aesculin and hippurate positive, acetoin negative. Grows at 45° but not at 15°. Ferments lactose, melibiose and arabinose, and usually raffinose, salicin and xylose. Tolerates 0·4% phenol and 4% NaCl, but not 10% bile or 8% NaCl. Acid from glucose 1–3 ml.

DISCUSSION

All strains examined fell into one of the two major divisions of the genus, homofermentative and heterofermentative, and were readily distinguishable by the production of gas from glucose in the latter case alone. The homofermentative division is further divisible upon the basis of growth at temperatures of 15° and 45° into two groups, whereas the heterofermentative division is relatively homogeneous in this respect. The three groups thus obtained correspond exactly to the three genera *Thermobacterium*, *Streptobacterium* and *Betabacterium* into which Orla-Jensen (1919) proposed to divide the lactobacilli. It was considered, however, on the basis of the present findings, that the last two should each be divided into two, and that the resulting five groups were then of equal taxonomic rank, and were of the order of species of *Lactobacillus*. The further subdivision of some of these groups into a larger number of smaller species is not considered to be taxonomically desirable and these have been relegated to the rank of varieties. An infinity of subdivisions may be made in any biological group, if each least variant character is taken into consideration, and this is especially true of *Lactobacillus*.

The present classification has been based upon the results of the physiological tests described, and their comparison with those of other workers. It is considered that the high degree of correlation between these, and the morphological characters of the organisms, provides a remarkable confirmation of the validity of the system (Davis *et al.* 1955).

Many of the tests used were originally devised for the classification of the closely related streptococci. Aesculin hydrolysis (Orla-Jensen, 1934), hippurate hydrolysis (Ayers & Rupp, 1922), and ammonia production from arginine (Niven, Smiley & Sherman, 1942) are good examples. Refinement of technique improves the value of these tests, e.g. the hydrolysis of aesculin in the present form provides an excellent diagnostic test, whereas the method used by Rogosa *et al.* (1953) often produced 'delayed and equivocal' results, and neither these workers nor Wheater (1955*a*) obtained the clear distinctions recorded in this case.

Although Rahe (1918) reported the common occurrence of the gas-producing *Bacillus acidophil-aerogenes* in human saliva, subsequent studies on oral lactobacilli usually omitted any reference to this important character, and its validity was even denied (Whyte, 1942; Stralfors, 1950), until the recent report by Tilden & Svec (1952). Comparison of the gas detection methods of Gibson & Abd-el-Malek (1945), Tilden & Svec (1952) and the Durham tube method of this study, showed that all three gave exactly the same results; but the latter was adopted because of its greater simplicity both in preparation and use.

It was frequently found that supposed variant colonies, selected during the course of purification, were eventually discovered to be exactly like the parent cultures. In some cases the variations were of the type reported by Rogosa *et al.* (1953), but more commonly they were merely due to the fact that culturally young and crowded colonies of certain species differed in form from older, discrete colonies. This is a point of some importance in consideration of the reliability of colony characteristics as a taxonomic criterion. The colony forms recorded above as the normal type for each group agree with the findings of Rogosa *et al.* (1953) and help to clarify the earlier reports of Rahe (1914), Rettger & Horton (1914), Morishita (1929) and Hadley, Bunting & Delves (1930), among many others who described this feature.

The relationship between *Lactobacillus acidophilus* and *L. bulgaricus* has long been acknowledged. Their present designation as varieties of one species is considered logical in view of their very close resemblance, as also is the inclusion of the *L. lactis* strains of Sharpe (1955). Other so-called species, e.g. *L. helveticus* (*Bergey's Manual*, 1948) are probably also varieties of this group. Members of the variety acidophilus of *L. acidophilus* are the only strains of any species which appear to be restricted to a parasitic habitat, in this case, the mouth.

It is clear that the *Lactobacillus helveticus* of Wheater (1955*b*) is not the same as that described in *Bergey's Manual*. The former represents the *L. casei* var. *rhamnosus* of Rogosa *et al.* (1953) and the *L. casei* var. A of the present study, while the Bergey species is clearly a member of the high-temperature group, or *Thermobacterium* of Orla-Jensen (1919) and the *L. acidophilus* of the present study. The characters quoted by Rogosa *et al.* (1953, Table 1) agree with the second of the above groupings. It is therefore apparent that the name helveticus has been accorded to quite different organisms, presumably on a basis of origin (from Swiss cheese), exactly as many authorities have tended to identify any lactobacillus from the mouth as *L. acidophilus*. The converse of this fallacious systematic method is found when essentially similar organisms from different sites are accorded different names. The history of classification of lactobacilli abounds with examples of both.

It is true that the members of the genus *Lactobacillus* are divisible into both major and minor groups, distinguished by characters of greater or less relative importance. This was recognized by Orla-Jensen (1919) when he defined his three genera with their constituent species. But since the compression by Winslow *et al.* (1920), of Orla-Jensen's three genera into one, *Lactobacillus*, the tendency has been to define any distinguishable groups as species, with little regard for the significance of the characters employed. Thus the species concept has been of very unequal status in different systems of classification. Rogosa *et al.* (1953), whose classification system is very like that described here, but more complex in structure and application, showed that nutritional requirements and lactic acid isomer production were in excellent accordance with the morphological and physiological characters employed here.

Some confirmation is also obtainable from the serological groupings of Sharpe (1955), which confirm the close relationship between *Lactobacillus casei* and the *L. helveticus* of Wheater (1955*b*), and reflect certain other similarities between group 1 and group 5, which are apparent in the above definitions. The correlation between these various findings suggests that the relatively simple methods used in this study and by Wheater (1955*a*, *b*) are adequate for the purpose of classification in this genus.

Although noticeably more heterogeneous in physiological reactions than the homofermentative strains, the heterofermentative lactobacilli are nevertheless classifiable into two clear types. The *Lactobacillus buchneri* of Rogosa *et al.* (1953) was not identified as a separate species, but certain strains showing some characters intermediate between those of *L. fermenti* and *L. brevis*, e.g. hippurate hydrolysis, weak aesculin hydrolysis, which may represent this species, were encountered (*L. fermenti* var. E.). The lack of ammonia production from arginine by 19 of the 23 *L. brevis* strains examined is of considerable interest. Briggs (1953) recorded 11 arginine-negative heterofermentative strains in her group VII, and this character is so common as to be almost diagnostic for homofermentative strains.

The new species, *Lactobacillus cellobiosus* and *L. salivarius* named by Rogosa *et al.* (1953) from oral sites were not encountered in the present group of oral isolates.

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Studies on the Multiplication of a Tobacco Necrosis Virus in Inoculated Leaves of French-bean Plants

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SUMMARY: By treating leaves of French bean (*Phaseolus vulgaris* L.) in various ways at intervals after they were inoculated with the Rothamsted tobacco necrosis virus, a series of events in infected cells was detected and approximately timed. Treating leaves with ribonuclease, or floating them in water, in the first hour after inoculation decreases the number of infections; these treatments fail to do so later, perhaps because the virus has become firmly attached to some host component, or because cells injured at inoculation have healed and no longer allow virus particles to diffuse out or ribonuclease in.

After 2 hr. at 25° the virus is less readily inactivated by ultraviolet radiation than previously, suggesting that infected cells now contain more of substances that absorb radiation of 2537 A. After 4 hr. the inactivation curve with ultraviolet radiation deviates from the course of a first-order reaction, probably because some infected cells now contain more than one potentially infective particle. After 6 hr. the formation of some lesions cannot be prevented by irradiation, suggesting that newly formed virus has spread from initially infected cells to deeper tissues, where it is protected from inactivation. At 16° the mean time for virus to spread from the initially infected cells is 12·5 hr. and it probably spreads from some cells by 8 hr. and from most by 15 hr.

Newly formed virus is not detectable in extracts of inoculated leaves until after it has spread from the epidermis to deeper tissues. Up to 20 hr. after inoculation most of the extractable virus seems to come from the epidermis, but later it comes predominantly from other tissues.

When leaves of susceptible plants are rubbed with solutions of plant viruses, washed to remove surplus inoculum, and then macerated at intervals, there follows a 'latent' period during which the infectivity of successive samples of leaf sap not only fails to increase but usually decreases. With any given inoculum and host plant, provided the conditions are kept constant, the interval between inoculation and the time when new infective particles become detectable in the sap is reasonably constant, but the latent period differs with different viruses and hosts, and it also varies with changes in either the temperature or the virus content of the inoculum. With the Rothamsted tobacco necrosis virus in French bean, the virus-host combination with which we shall be mainly concerned, the minimum latent period at 22° is about 10 hr. (Harrison, 1955). Little or nothing is known about what goes on during this period, and the experiments we describe below, in which leaves were subjected to a variety of treatments at different times after they were inoculated, were made to gain some indirect evidence about the sequence of events between the application of the inoculum and the occurrence of new infective virus.

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METHODS

The inoculum was sap expressed from leaves of French bean (*Phaseolus vulgaris* L. var. Prince) infected with the Rothamsted tobacco necrosis virus (RTNV) and showing well-developed necrotic lesions. The sap was kept frozen, and samples were thawed and clarified by centrifuging at 6000 r.p.m. when needed. Inocula were diluted in distilled water. For experiments, and for assays by the local-lesion method, the primary leaves of French bean were used, and the apical shoots were removed before the plants were inoculated. Unless otherwise stated, plants were raised and kept during the period of an experiment in the fluctuating conditions of a glasshouse, with an average temperature of 20° and with no supplementary light. When experiments were made at a constant temperature, the plants were kept in a glass-sided incubator heated by four thermostatically controlled, electric strip-heaters. The air was kept moving by a fan hanging from the roof of the incubator, and the temperature did not vary by more than 1° from the mean.

Leaves were inoculated by rubbing their upper surfaces as evenly as possible with the forefinger wetted with inoculum. When 'Celite', a diatomaceous earth sold by Johns-Manville, was used to increase the number of lesions produced by an inoculum (Kalmus & Kassanis, 1945), it was suspended in the inoculum. Infectivity assays were made by the local-lesion, half-leaf method. When several inocula or treatments were to be compared, they were distributed so that each occurred on at least six half-leaves, and equally often on left- and right-hand halves.

Two sources of ultraviolet radiation (UV) were used, both low-pressure mercury discharge lamps. One, lamp *B*, made by the Thermal Syndicate Ltd., was fitted with a chromium-plated, cylindrical reflector; the intensity of radiation, most of which was wavelength 2537 A, at 20 cm. was 870 μW/sq.cm. The other, lamp *W*, a tube 60 cm. long made by Hanovia Ltd., who state that over 95% of the radiation is of wavelength 2537 A, was fitted with an ozone-reducing filter; at 20 cm. from the tube it produced similar effects to lamp *B* in about half the time. All leaves were exposed at 20 cm. from the tubes.

Crystalline ribonuclease was prepared from beef pancreas by Kunitz's (1940) method.

RESULTS

The effects of exposing inoculated leaves to ultraviolet radiation

As ultraviolet radiation can destroy the infectivity of plant viruses and also the ability of plant cells to support the multiplication of viruses (Bawden & Kleczkowski, 1952, 1953; Benda, 1955), irradiation of recently inoculated leaves might prevent lesions from forming either because the infecting virus was inactivated or because the host cells were affected. For various reasons, we think that most of the lesions which failed to develop in our experiments did so because the virus was inactivated and that probably few cells were so affected by the radiation that they could not support virus multiplication and yet still contain infective virus.

First, exposures to the same amount of radiation decrease the numbers of lesions much more when they are given shortly after than when given immediately before inoculation. For example, giving exposures of 0·5, 1, 2 and 3 min. with lamp *B*, the lesions formed on irradiated half-leaves, expressed as a percentage of those on the unirradiated halves, were 50, 29, 3 and 1 when the leaves were exposed after inoculation, and 80, 80, 63 and 17 when they were exposed before inoculation. Secondly, when the logarithms of such percentages are plotted against the time of exposure to UV, the lines for exposures after inoculation are straight, as they are for the inactivation of virus preparations by UV *in vitro*, typical of the course of a first-order reaction and often called a 'single-hit' process. By contrast, the lines for exposures before inoculation are curved and deviate considerably from the course of a first-order reaction (Fig. 1).

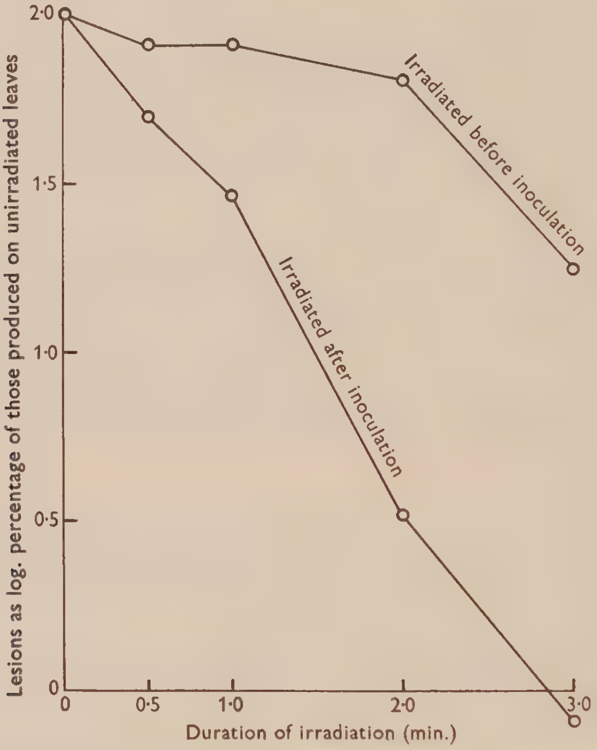

Fig. 1. Effect on numbers of lesions of irradiating French-bean leaves, for various times with lamp *B*, before and after they were inoculated with the Rothamsted tobacco necrosis virus.

Thirdly, if effects on the host plant were comparable to those on the infecting virus, irradiation of leaves after inoculation should decrease lesion numbers similarly, regardless of the sensitivity to UV of the virus used; but this is not so. When we inoculated leaves of *Nicotiana glutinosa* with two strains of tobacco mosaic virus that differ considerably in the ease with which they are inactivated *in vitro* by exposure to UV (Siegel & Wildman, 1954; Bawden & Kleczkowski, 1955), and then exposed the leaves to equal amounts of irradiation, proportionally many fewer infections were obtained with the more sensitive strain U2 than with the type strain.

Effects on the host plant may have contributed something to the total decrease in numbers of lesions when inoculated leaves were given the longer exposures to UV, but this contribution was probably too small to prevent the decrease from being used to estimate the inactivation of the infecting virus. To mitigate damage to the leaves and to stop them from becoming bronzed, whenever possible they were kept in daylight for some hours immediately after they were irradiated. With our exposures, damage to the host probably did not extend deeper than the epidermis. Benda (1955) found that one-third of the incident radiation of 2537 A could be transmitted by pieces stripped from the upper epidermis of French bean leaves, but less than this will enter the palisade cells and much of what does is probably absorbed by the first layer of chloroplasts. With the largest exposures to UV that we used, although the epidermis dies when the leaves are kept in the dark, the palisade cells not only survive but seem unharmed (Bawden & Kleczkowski, 1952). Also, when the upper surface of leaves is inoculated with RTNV and the lower surface is then exposed to much more radiation than is needed to kill its epidermis, numbers of infections are unaffected.

On the assumption that inoculation introduces virus only into epidermal cells, and that only virus in these cells is affected by exposing leaves to UV, experiments were done to find how long the inoculated virus takes to multiply in the epidermis and to spread from there to deeper cells. Fig. 2 gives the combined results of two experiments at 16°, in which leaves were exposed to 3 min. radiation from lamp *B* at different intervals after they were inoculated. The virus seems to have spread from some sites of infection within 8 hr. of inoculation and from 80 % within 15 hr. The average time taken for newly formed virus to enter the mesophyll from the initially infected epidermis can be taken as the interval between inoculation and when half the lesions that can be suppressed by irradiation immediately after inoculation form despite irradiation. Fig. 2 shows that the leaves irradiated immediately after inoculation produced 2 % of the lesions produced by unirradiated leaves, so that the 50 % suppression time was when irradiated leaves produced 51 % of the number of lesions formed on the controls, which was 12·5 hr. after inoculation.

This 50 % suppression time varies with the temperature; at 25° it is 6 hr. for RTNV in French bean. It also differs with different viruses and host plants; with RTNV it is about twice as long in tobacco as in French bean; at 25° it is 5·5 hr. for tomato aucuba mosaic virus in tobacco and in *Nicotiana glutinosa*, and it is about 13 and 19 hr., respectively, for tomato bushy stunt and tomato spotted wilt viruses in *N. glutinosa*. When leaves are exposed to the same amount of radiation immediately they have been inoculated, the proportion of lesions that develops is smaller with tomato spotted wilt virus than with RTNV or tomato bushy stunt virus, and smaller with these than with the tomato aucuba mosaic or the type strain of tobacco mosaic virus. The results with the last four agree with their relative sensitivities to UV when irradiated *in vitro* (Bawden & Kleczkowski, 1953, 1955), from which it seems that tomato spotted wilt is the most sensitive of the viruses used.

To gain some information about changes in epidermal cells before newly formed virus moves out of them, experiments were made of the type described by Luria & Latarjet (1947) with coliphages, and half-leaves were exposed to four doses of UV (20, 40, 80 and 160 sec. with lamp *W*) at different times after they were inoculated with RTNV. In each experiment, twelve batches of plants were inoculated and kept at 25°. The leaves were washed after inoculation. At three different times, four batches were removed from the incubator and one batch given each of the exposures to UV. The leaves were detached, and as some were still wet from washing, all were dipped in water, and half of each was irradiated: wetting did not influence the effects of irradiation. The times when leaves were inoculated were so arranged that the intervals between inoculation and irradiation were the same for leaves receiving each of the four doses of UV. After irradiation, the leaves were floated on water until the lesions developed.

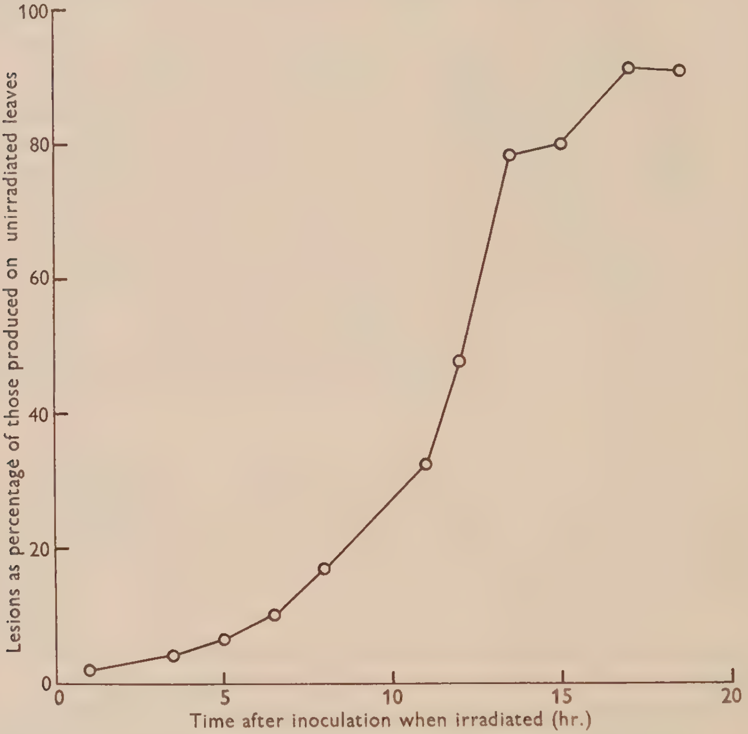

Fig. 2. Effect on numbers of lesions of irradiating French-bean leaves, for 3 min. with lamp *B*, at different intervals after they were inoculated with Rothamsted tobacco necrosis virus and kept at 16°.

The average number of lesions as a percentage of the unirradiated controls was calculated for each treatment in each experiment, and the averages for all experiments are given in Table 1. The average numbers of lesions per half-leaf on irradiated and control half-leaves were also calculated for each treatment, and were used to calculate the proportion of the virus that survived when the inoculated leaves were irradiated. Lesions counts, unlike plaque counts with phages, cannot be used directly for this purpose because the number of lesions produced by inocula is not linearly related to their virus content. To obtain values more representative of virus survival, the numbers of lesions given by any treatment and by its unirradiated control were interpolated on a curve obtained by plotting numbers of lesions obtained with inoculum applied at a range of dilutions, against the logarithm of the dilution. By finding what difference in virus content was needed to produce the same fall in lesion numbers as that produced by irradiation, the proportion of surviving active virus could be calculated. For example, if forty lesions were produced by the control and twenty by the irradiated leaves, the dilution curve showed that, to get this same result by dilution would require the inoculum to be diluted 1 in 2·46. In this example, then, where the irradiated leaves produced half as many lesions as the controls, the percentage of inoculated virus that survived irradiation was 100/2·46 = 40·6. The average numbers of lesions per half-leaf produced with every treatment in each experiment were so transformed and are given in Table 2.

Table 1. *The effect on lesion number of exposing French-bean leaves to various amounts of ultraviolet radiation at different times after inoculation*

Period after inoculation (hr.)	Ultraviolet dose (sec.) 20	40	80	160	No. of experiments
	Number of lesions (%)*				
0	53·60	21·14	4·70	1·22	5
1	50·83	23·20	5·02	1·65	6
2	69·79	49·66	29·40	6·59	9
3	75·37	48·93	35·10	6·52	3
4	76·30	66·93	48·63	13·22	8
5	89·3	78·7	52·7	38·3	1
6	87·30	82·70	62·66	50·32	5
7 and 8	81·22	91·88	80·12	76·44	5

* The numbers of lesions formed on irradiated leaves expressed as the percentage of the number that formed on the unirradiated controls.

Inoculum was infective sap at 1/100, and plants were left at 25°.

Fig. 3 shows the lines obtained when the logarithms of the percentages of virus that survived irradiation at different times after inoculation are plotted against the UV dose. Except for differences in the time scale, these lines resemble those given by coliphage T7 (Benzer, 1952). The lines for 5 min. and 1 hr. after inoculation follow the same course and both are straight up to 80 sec. irradiation. No significance is attached to the deviation from straightness at 160 sec., for only 1% of the initial virus was then surviving and this may have survived because it was in cells that were for some reason shaded from the radiation. A straight line would be expected if irradiation prevented

Table 2. *The proportion of infecting virus surviving in leaves given different ultraviolet doses at different times after inoculation*

Period after inoculation (hr.)	Ultraviolet dose (sec.)				No. of experiments
	20	40	80	160	
	Number of lesions (%)*				
0	43·70	16·40	3·46	0·89	5
1	37·70	16·26	2·89	1·02	6
2	62·09	40·34	22·30	4·22	9
3	65·23	37·00	25·47	4·67	3
4	67·76	58·04	38·88	8·25	8
5	80·5	66·8	38·3	22·4	1
6	83·38	74·26	51·68	39·82	5
7 and 8	72·24	87·00	69·22	67·38	5

* The numbers are calculated from the lesion totals given as averages in Table 1, and transformed into the percentage of surviving virus by interpolation of a dilution curve.

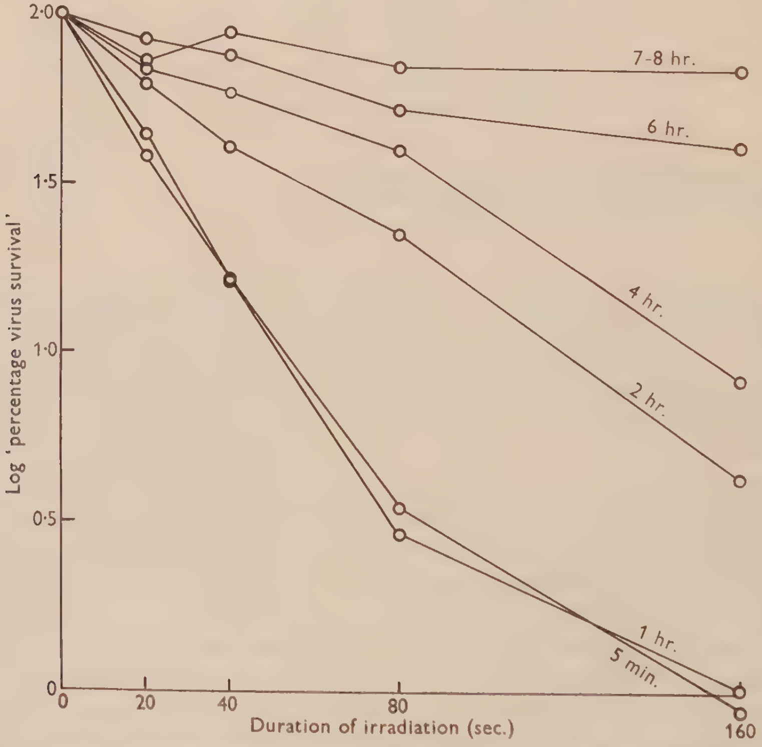

Fig. 3. Effect on the percentage of surviving virus of irradiating French-bean leaves, for various times with lamp *W*, at different intervals after they were inoculated with the Rothamsted tobacco necrosis virus and kept at 25°.

lesion formation by inactivating one virus particle in each infected cell. The results suggest that, with inocula of the type used, most infections are caused by single particles. Any change that these single particles undergo during the first hour after inoculation seems not to affect their sensitivity to UV.

Except for a suggestion of flattening between 40 and 80 sec. irradiation, the line at 2 hr. after inoculation is still essentially straight, but the slope is less steep than at 1 hr. It seems that there is still only one virus particle/cell, but that it has become more difficult to inactivate by UV. This might have various explanations. The virus may have changed to a form more resistant to inactivation, or it may have combined with some host component that absorbs UV and protects it from the radiation. Alternatively, the infected cell may have synthesized substances that absorb UV; by analogy with infection by coliphages, which early leads to synthesis of nucleic acid, it is plausible to assume that the most likely substances are purines and pyrimidines. We think it unlikely that effects of UV on the ability of the host cells to sustain virus multiplication played any significant part in determining the course of this or any of the lines, but unfortunately there seems no way of distinguishing whether host sensitivity to UV is affected by the processes leading to virus synthesis. All that can be said is that UV exposures of 20 and 40 sec., which have no effect on the number of infections when given before inoculation, considerably decrease it when given up to 2 hr. after inoculation, and the line obtained at 2 hr. is not curved like that obtained for irradiation before inoculation (Fig. 1).

From 2 to 4 hr. after inoculation the resistance to inactivation by UV again increases and the 4 hr. line is not straight. The curvature shows only when leaves are irradiated for more than 20 sec., which suggests that, at some sites, inactivation of single infective particles still suffices to prevent a lesion from developing, but that, at others, more than one now have to be inactivated. Again, effects on the host cells may be influencing the curve, but that some cells probably contain more than one infective, or potentially infective, particle by 4 hr. after inoculation is indicated by the effects of the later irradiations. The 6 hr. line is also curved, but bends in the opposite way from the 4 hr. line; prolonging the exposure to UV from 80 to 160 sec. has little extra effect, showing that irradiation can no longer prevent lesions from forming at many infection sites. We take this fact as evidence that newly formed virus has spread to cells below the epidermis, and the results agree with the time estimated for this occurrence by the experiments using a single dose of UV. The failure of prolonged irradiation to prevent lesion formation shows even more obviously in the 7–8 hr. line, which suggests that, by this time at 25°, virus has entered the mesophyll from about 80 % of the initial infection sites.

To gain information about the main sites of virus formation at later stages of infection, leaves were irradiated at intervals between 16 and 40 hr. after they were inoculated. These tests required infectivity assays on extracts from macerated leaves and they were not begun until 16 hr. after inoculation because until then the extracts contain too little virus to be assayed accurately. French-bean leaves were rubbed over their whole surfaces with infective sap diluted 1/100, washed thoroughly, and at various intervals the inoculated surfaces of eight half-leaves were irradiated for 3 min. from lamp *B*. Immediately after irradiation, the terminal halves of the leaves were cut off, and the irradiated and unirradiated quarters were separated by cutting out the mid-rib. The eight irradiated quarter-leaves were pooled, macerated and the sap expressed and frozen: the unirradiated quarter-leaves were treated in the same way. At the end of an experiment, the samples of sap were thawed, clarified by centrifuging, and their infectivities compared. The basal halves of the leaves were left on the plants to observe how irradiation affected lesion formation. Irradiation at 16 hr. usually slightly decreased the number of lesions, but not after 19 hr. Lesions that developed on irradiated parts were often smaller than others; they began to appear about 40 hr. after inoculation and if irradiated then the usual black lesion was surrounded by a brown halo.

The results of four experiments are given in Table 3, where the numbers of lesions produced by samples of sap from the irradiated quarters of leaves are expressed as the percentages of the numbers produced by the unirradiated controls. Although these numbers will not exactly reflect the relative virus contents of the two lots of sap, they will serve as approximations. The differences between different experiments probably reflect the fact that the temperatures were not controlled, but the general trend is clear. At 16 hr. most of the newly formed virus is in the epidermis, but by 19 hr. a half is coming from the mesophyll. Between 19 and 24 hr. the proportion of the total extractable virus that is inactivated by exposing leaves to UV changed only slightly, suggesting that during this period virus was multiplying equally in epidermal and other cells. By 40 hr., however, irradiating inactivates only a small proportion of the total extractable virus; this probably means that the main suppliers of virus are now deeper cells, but is not wholly conclusive because lesions are now forming and the black necrotic cells may absorb UV and protect virus particles from inactivation.

The increase in virus content of the control leaves can be estimated in arbitrary units by finding at what dilution the samples of sap obtained at different times give similar numbers of lesions, and then multiplying the numbers of lesions by the dilution. For Expt. D in Table 3, for example, the relative virus contents at 16·5, 19·5, 21·5 and 24 hr. after inoculation were, respectively, 155, 427, 3055 and 15425 units, of which 119, 189, 1326 and 5553 could be inactivated by irradiating leaves. This suggests that, during this period, virus continued to be produced in the epidermis, but that an increasingly large proportion of the total came from deeper cells as the infection proceeded. Combining the results from various tests, it can be concluded that for the first 8 hr. after inoculation new virus is produced only by the epidermal cells infected at inoculation; subsequently, it may continue to come from these, but is also now beginning to come from nearby cells, probably both epidermal and mesophyll. There is no evidence to suggest that any one type of leaf cell produces more virus than any other, and that most ultimately comes from cells other than the epidermis is to be expected because there are more of these other cells in the leaf. In seeming to occur more or less equally in all leaf cells, RTNV resembles tobacco mosaic virus in systemically infected tobacco leaves and differs from two aphid-transmitted viruses, cabbage black ringspot and henbane mosaic, with which most of the extractable virus seems to be contained in the epidermis (Bawden, Hamlyn & Watson, 1954).

Table 3. *The proportion of tobacco necrosis virus unaffected by ultraviolet radiation in leaves at different times after inoculation*

Expt.	Time of irradiation (hours after inoculation)					Average temperature (°)
	16·5	19·5	21·5	24·0	40·5	
	Number of lesions (%)*					
A	4·0	32·0	52·8	60·4	77·1	19·5
B	28·9	52·4	19·1	60·1	98·4	19·0
C	18·0	53·5	54·5	53·7	76·5	21·5
D	23·5	55·7	56·6	64·0	97·4	22·0
Average (X)	18·6	48·4	45·8	59·6	87·4	—
$100-X$	81·4	51·6	54·2	40·4	12·6	—

* The numbers of lesions produced by sap from irradiated leaves expressed as percentages of those produced by sap from control unirradiated leaves.

The inoculum was infective sap at 1/100, which produced about 75 lesions per half-leaf.

Treatment of inoculated leaves with pancreatic ribonuclease

Many substances which do not destroy plant viruses *in vitro* when incorporated in inocula prevent the viruses from infecting their host plants. Ribonuclease is one of the most powerful of these inhibitors of infection and, at 5 mg./l., it prevents potent preparations of RTNV from causing any lesions in French-bean leaves. To gain some information about the phase of infection that the enzyme affects, we have treated inoculated leaves with it at various intervals after inoculation. The leaves were treated in two ways, either rubbed with 50 mg./l. solutions of ribonuclease, or immersed for 15 sec. in the enzyme solution. As controls, comparable leaves were treated similarly with water. Neither of the methods of applying ribonuclease after inoculation decreased numbers of infections nearly as much as does incorporating the enzyme in the inoculum, possibly because they did not introduce the enzyme into all infected cells, which almost necessarily occurs when mixtures are inoculated, because the enzyme and virus combine *in vitro*. On average, rubbing with solutions of the enzyme at 5, 15, 30 and 60 min. after inoculation decreased the numbers of lesions to 19, 40, 42 and 90 % of those produced on the control leaves, and immersing in the solutions decreased them to 10, 21, 37 and 80 %. The greater effect of immersing may be apparent rather than real, because rubbing with water alone decreased the numbers of lesions more than did immersion in water.

Clearly, ribonuclease can prevent infections if it is applied to leaves soon after they are inoculated and its ability to do so decreases rapidly with increasing time after inoculation, but it is less clear how to interpret this behaviour. One obvious interpretation is that the enzyme affects only a very early stage in the processes that lead to virus multiplication, a stage that has ended before any change is detectable in the resistance of the infecting particles to UV. The time during which the enzyme has any large effect is approximately the same as that needed for particles of potato virus X that have been inactivated by UV to reach the state in inoculated leaves where their ability to multiply can be restored by visible light (Bawden & Kleczkowski, 1955). By analogy with the behaviour of infecting coli phages, this early stage might be, as suggested by Casterman & Jeener (1955) for tobacco mosaic virus, the nucleic acid separating from the protein part of the virus and so becoming susceptible to hydrolysis by ribonuclease; or it might be the attachment of the virus particles to some specific component of the host cells, and that this attachment is prevented by ribonuclease, as it seems to prevent the attachment of a Rhizobium phage to its host (Kleczkowski & Kleczkowski, 1954).

There is, though, another equally plausible interpretation for the fact that ribonuclease acts for only a short time after inoculation, namely, that for only a short time after inoculation are cells permeable to it. Ribonuclease is a large molecule and, like virus particles, will probably not readily penetrate uninjured leaves. Also, the length of time after inoculation during which ribonuclease decreases lesion numbers is about the same as that during which rubbed leaves remain susceptible to infection by suspensions of virus sprayed over them. For example, in experiments in which French-bean leaves were rubbed with suspensions of 'Celite' in distilled water and then sprayed at intervals with infective sap at 1/20, the numbers of lesions formed on 12 half-leaves sprayed at 0·1, 0·25, 0·5, 1, 2, 4 and 24 hr. after rubbing were 3355, 2702, 2165, 873, 87, 32 and 7. Thus the susceptibility of the rubbed leaves to infection fell greatly during the first hour, and if this reflects the repair of wounds to the cells and the restoring of a barrier to the entry of virus particles, the same repair may also prevent the entry of ribonuclease.

Effect of floating and rubbing inoculated leaves

To assess the extent to which ribonuclease decreased infections when applied to inoculated leaves, comparisons had to be made with leaves treated comparably with water, for floating in water or rubbing with the forefinger wet with water also decreases the numbers of lesions produced by RTNV in French-bean leaves. The effect of floating is less than with *Nicotiana glutinosa* leaves inoculated with tobacco mosaic virus and it also operates for a shorter time after inoculation (Bawden & Kassanis, 1954). The total lesions from three experiments in which leaves were floated in water with their inoculated surfaces upwards at intervals of 0·1, 0·5, 1, 2, 4 and 8 hr. after inoculation were 1477, 1913, 2490, 2803, 2886 and 2952. Washing freshly inoculated leaves in water also decreases the numbers of lesions that form, but this effect is not additive to the one obtained by floating continuously in water. Both probably act by removing particles from cells in which they otherwise would have remained and multiplied, and little RTNV seems to be removable after the first 30 min. Whether the virus particles have by then become firmly attached to some component of the host cell, or whether the permeability of the cells has decreased by the healing of wounds, cannot be concluded.

Rubbing leaves with the forefinger wet with water decreases numbers of lesions when done at longer intervals after inoculating the virus. When rubbed at 0·1, 1, 2, 4, 6 and 8 hr. after inoculation, leaves produced 39, 63, 75, 83, 86 and 97 % of the number of lesions produced on unrubbed leaves. Quarter of the total effect of rubbing is still obtained at 4–6 hr. after inoculation; the results of the experiments exposing inoculated leaves to UV suggest that this is the period when new virus particles are beginning to form and to invade deeper cells. Until this has happened, rubbing seems still able to prevent a lesion from developing. It presumably does so by injuring (perhaps killing) infected epidermal cells so that virus particles can no longer be synthesized in or move from them.

Table 4. *The number of lesions in French-bean leaves inoculated once (V) or twice (VV) with a tobacco necrosis virus, and rubbed with water before (WV) or after (VW) inoculation*

<table>
<tr><th>Expt.</th><th>V</th><th>VW</th><th>WV</th><th>VV</th></tr>
<tr><td>A</td><td>553</td><td>492</td><td>488</td><td>754</td></tr>
<tr><td>B</td><td>141</td><td>108</td><td>90</td><td>173</td></tr>
<tr><td>C</td><td>2050</td><td>418</td><td>312</td><td>989</td></tr>
<tr><td>D</td><td>356</td><td>119</td><td>63</td><td>196</td></tr>
<tr><td>E</td><td>510</td><td>335</td><td>359</td><td>835</td></tr>
<tr><td>F</td><td>98</td><td>71</td><td>59</td><td>127</td></tr>
<tr><td>G</td><td>429</td><td>459</td><td>494</td><td>743</td></tr>
<tr><td>H</td><td>147</td><td>106</td><td>118</td><td>217</td></tr>
<tr><td></td><td>4284</td><td>2108</td><td>1983</td><td>4034</td></tr>
<tr><td></td><td></td><td colspan="2">4091</td><td></td></tr>
</table>

The numbers are the total lesions produced on 10 leaves. The leaves were not washed after inoculation and the interval between any two successive treatments was 15 min. or less.

This injurious effect of rubbing is of some interest because rubbing the leaf surface with virus preparations is the normal method of getting infections. It is usually assumed that the rubbing serves the purpose of wounding the epidermis and allowing virus particles to enter the cell. It seems, though, that at least two kinds of wounds are formed, and that all wounds do not favour the establishment of infection. Table 4 gives the results of experiments testing the effects of inoculating French-bean leaves once (V) or twice (VV) with RTNV and inoculating once either preceded (WV) or followed (VW) by a rub with water. Treatments (WV) and (VW), with one exception, gave fewer lesions than (V), and the same number of lesions was obtained whether the rub with water preceded or followed inoculation. This clearly suggests an effect on the host, rubbing destroying potential sites of infection, whether or not they already contain virus. In total, inoculating twice with the virus produced fewer lesions than inoculating once, though these two treatments produced very different results in different experiments. These differences seemed to be correlated with the condition of the plants when they were used. The leaves in Expts. C and D, when treatment (VV) produced many fewer lesions than (V), were thinner and more fragile than those used in the other experiments, when (VV) produced more lesions than (V). This suggests that the first inoculation affected the delicate leaves so that more of the possible sites of infection were made suitable for infection than in the tougher leaves, but that the second inoculation damaged many of these so much that they could no longer support virus multiplication.

The action of substances such as 'Celite' in increasing the numbers of lesions produced by a given inoculum is still imperfectly understood. It may do so, not simply because rubbing with it produces more wounds through which virus particles can enter, but because it injures cells so that their metabolism more often changes in a way that favours virus synthesis instead of preventing it. It seems unlikely that it acts by introducing virus into other cells than the epidermis and in which multiplication might occur more readily, for when we exposed leaves infected by inocula containing 'Celite' to UV, the formation of lesions was prevented as when leaves were inoculated without 'Celite'.

DISCUSSION

The results of our experiments clearly show a succession of events between the times when RTNV is inoculated to French-bean leaves and new virus becomes detectable in extracts from the leaves, but the nature of each event is less clear. Some results can be interpreted with reasonable assurance; for instance the change from a straight to a curved line in Fig. 3 most probably means that more than one potentially infective particle has appeared in most cells; also, the coincidence of the failure of UV and rubbing to affect lesion numbers can be taken as the time when virus particles have spread into the mesophyll. It is the earlier events that cannot be identified with any certainty, and the fact that they can be fitted into the sequence that occurs during infection by coliphages is suggestive, but no more than this.

As a first step in infection, coliphages are adsorbed by their hosts, and then the nucleic acid penetrates the cells. There is no direct evidence that plant viruses specifically combine with their host cells, or with any component of them; nor is there any evidence that infection necessitates the nucleic acid separating from the protein moiety. Obviously such a sequence of events is compatible with our results from floating inoculated leaves in water and treating them with ribonuclease, treatments that can prevent infection if applied shortly after leaves are inoculated but that soon become ineffective. There is, though, no need to postulate a specific attachment of the virus that prevents it from dissolving, or a fleeting period during which the nucleic acid becomes susceptible to ribonuclease. Our results are as easily explained by assuming that, during inoculation, the host cells are made permeable to large particles like viruses and ribonuclease, but that, provided the damage is not so severe that the cells die, they soon repair the wounds and regain their usual impermeability to large particles.

The increase in resistance of the infecting virus to inactivation by UV at about 2 hr. after inoculation is again explicable by analogy with coliphages, for infection with these early leads to an increase in the nucleic acid content of the infected bacteria. There is no direct evidence that infection with RTNV increases the nucleic acid content of infected cells, but Commoner (1954) states that the insoluble nucleic acid in tobacco leaves increases before tobacco mosaic virus occurs in quantity and then decreases as the virus increases. The increase is not detectable until several days after the leaves are inoculated, but this difference in time scale from ours need not mean different phenomena. Our results are concerned only with changes in the few epidermal cells that become infected at inoculation, whereas to show differences in extracts from leaves means waiting until a considerable proportion of all the leaf cells has become infected. However, the increased resistance of RTNV to UV could have other explanations. For example, if the infecting virus does combine with, or enter, some cell components, say, mitochondria or plastids, which are rich in nucleic acid or some other substance that absorbs radiation of 2537 A, then the virus would be protected from the radiation.

It is of some interest that new virus seems to be able to invade other leaf cells from those infected at inoculation long before it becomes detectable in extracts of macerated leaves. This could have several explanations. Palisade cells may be more easily infected than epidermal ones. Or the particles as first formed may differ from later forms, and may be able to infect cells within a leaf but lack something that makes them stable *in vitro*. Or it may be that there are enough specific adsorption sites nearby to fix the first particles as soon as they are formed, so that they never become freely extractable in sap. It is equally likely, however, that the interval between the time when virus becomes able to invade other cells in a leaf and the time when it becomes detectable in leaf extracts simply reflects the crudity of our inoculation methods. Spread from cell to cell may need only one virus particle, whereas, to get a single lesion, we need to apply 1 ml. of inoculum containing about 10^5 particles and the lag may simply be the time taken for this concentration to be reached.

This work was done while one of us (B. D. Harrison) was a research student of the Agricultural Research Council.

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The Mode of Action of Antibiotic Synergism and Antagonism: the Effect *in Vitro* on Bacteria not Actively Multiplying

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SUMMARY: The synergistic effect of certain pairs of antibiotics might be due to action on 'persisters'; i.e. members of a bacterial population which survive exposure to single drugs, perhaps while in a state of reduced metabolic activity, although their offspring do not show enhanced resistance. Hence, antibiotics were tested *in vitro* under conditions minimizing bacterial multiplication and metabolism. At 37°, neomycin, polymyxin, and streptomycin were more effective in the absence of nutrients than in nutrient broth; oxytetracycline and bacitracin were equally effective in either environment; but penicillin had little effect in the absence of nutrients. At 4°, bactericidal action varied with the test organism so that differences could not be attributed solely to the 'resting' state of the bacteria. Conditions not permitting bacterial multiplication prevented synergism and those antagonisms demonstrable in broth. Probably synergism depends upon rapid bactericidal action preventing the emergence of persisters rather than upon destruction of these relatively inactive forms.

Most studies on the action of antimicrobial drugs *in vitro* have been made with multiplying bacteria, and the effect of combinations of antibiotics under conditions unfavourable to bacterial growth has not been investigated intensively. Reports on the effectiveness of antibiotics in environments which do not permit active bacterial multiplication seem contradictory, but some of the apparent disagreements are probably due to differences in the techniques used and in the bacteria tested. Thus far, synergism and antagonism among antibiotics have been observed only under conditions which permit growth and multiplication (Jawetz & Gunnison, 1953). Information about the action of antibiotics on non-multiplying bacteria is particularly needed with regard to so-called 'persisters', a term applied by Bigger (1944) to those members of bacterial populations which survive the action of antimicrobial drugs, although their offspring are no more resistant to the drugs than the original parent culture. A possible explanation for this phenomenon is that persisters may be in a phase of relative metabolic inactivity. As expressed by Davis (1954), '...these cells appear to be genotypically drug-sensitive but phenotypically drug-resistant'. In some chronic disease states, persisters may explain the failure of chemotherapeutic agents to eradicate infection. In certain chronic infections, synergistic drug combinations have resulted in cure, whereas single drugs, even though exhibiting antibacterial action, have failed. Synergism can be measured as a function of the bactericidal rate of a combination of drugs against a given organism as compared with the rate of each component. The question arises whether the curative effect of a synergistic combination might be attributed to its ability to kill persisters or to prevent their emergence. To elucidate this problem, antibiotics were tested *in vitro* under conditions minimizing growth, metabolism and multiplication.

METHODS

Antibiotics. Bacitracin, neomycin sulphate, and crystalline potassium penicillin G, dihydrostreptomycin sulphate, polymyxin B sulphate, and oxytetracycline hydrochloride (Terramycin) were obtained as sterile dry powders. These were dissolved in sterile double glass-distilled water in concentrated solution and stored at 4°. Dilutions in the respective diluents were prepared aseptically just before use.

Diluents. Dilutions of drugs and suspensions of bacteria were prepared in sterile double glass-distilled water or in Ringer's solution. For certain tests, imidazole buffer solution was prepared by adding 1·75 g. imidazole to 90 ml. 0·1 N-hydrochloric acid and making up to 100 ml. with distilled water. This stock solution was diluted 1/10 and sterilized in the autoclave (pH *c.* 7·2).

Media. Bacteria were grown in broth composed of proteose peptone No. 3 (Difco), 20 g.; glucose, 0·5 g.; sodium chloride, 5 g.; disodium phosphate ($Na_2HPO_4.12H_2O$), 5 g.; water, 1000 ml.; pH 7·2. Proteose no. 3 agar (Difco) was used for plate counts.

Bacteria. Streptococcus faecalis (strain 16), *Staphylococcus aureus* (strains Heatley and De), *Klebsiella pneumoniae* (strain AD), and *Escherichia coli* (strain Da) were used.

Procedure. The bacteria were inoculated into broth and incubated at 37° for 18 hr. The organisms were then washed twice in the respective diluent and uniformly resuspended therein to give a turbidity equal to that of the original 18 hr. culture. Dilutions of antibiotics were added to tubes of sterile glass-distilled water, Ringer's solution, or imidazole buffer. Each tube was then inoculated with the washed suspension of the test bacteria to give 10^6–10^8 organisms/ml. in a total volume of 15 ml. Similar tubes containing broth were inoculated with the unwashed bacterial culture for comparison. Samples were withdrawn at 0, 4, 7, 24, and 30 hr. of exposure at either 4° or 37° and the number of viable organisms was determined by the usual plate count procedure. Differences between counts of less than tenfold (1 log unit) were regarded as insignificant.

Definitions. The 'minimal bactericidal dose' is defined as the smallest amount of antibiotic which resulted in a bacterial count significantly lower than that of the drug-free control at the time of sampling and also lower than the initial count immediately after inoculation in at least two successive determinations within 30 hr. of exposure to the drug. 'Synergism' is defined as follows: 'The addition of one drug to another results in a marked increase in bactericidal rate within the first 8–24 hr. of exposure *in vitro*, and the bactericidal rate of the combination is more rapid than that of twice the concentration of each single drug participating in the mixture' (Jawetz & Gunnison, 1953). 'Addition' refers to a somewhat more rapid bactericidal rate with a combination of drugs than with either drug alone but no more rapid than that of twice the concentration of the more active single drug alone or than that of the optimal dose of drugs showing a zone effect. 'Indifference' means that the bactericidal rate of a combination is the same as that of the more active drug alone. 'Antagonism' is defined as a less rapid bactericidal rate with a combination than with the more active drug alone.

RESULTS

Effects of single antibiotics

The bactericidal effect of the various antibiotics on washed bacteria suspended in distilled water at 37° as compared with their effect in nutrient broth is summarized in Table 1. In making such comparisons, it must be remembered that not only was there no demonstrable multiplication of the test bacteria in water but a significant number of the population of some of the test strains died within 24 hr., even in the absence of antibiotics (Fig. 1). Hence, the drug may merely accelerate the death-rate obtained in water alone, thus magnifying the apparent difference under the two conditions.

Table 1. *Effect of medium on bactericidal action of various antibiotics at 37°*

Organism	Minimal bactericidal dose* in											
	Neomycin		Polymyxin		Streptomycin		Oxytetracycline		Bacitracin		Penicillin	
	Broth	Water	Broth	Water	Broth	Water	Broth	Water	Broth	Water	Broth	Water
Streptococcus faecalis strain 16	>500	0·05	>500	5	500	20	20	5	2	10	5	>500
Staphylococcus aureus Heatley	25	0·5	500	<5	100	5	5	2	2	5	0·01	500
S. aureus De	10	1	>100	0·5	50	10	2	2	2	2	>500	500
Klebsiella pneumoniae AD	2	<0·5	0·5	0·05	5	1	5	5	>20	<5	1	>50
Escherichia coli Da	10	0·25	5	2	100	5	>20	10	100	100	>10	500

* As defined p. 510. Penicillin and bacitracin expressed in units/ml.; other drugs in μg./ml.

Neomycin, as previously reported (Gunnison, Kunishige & Jawetz, 1954), was more active in water than in broth. The difference in activity in the two environments varied with the test micro-organisms and was greatest with *Streptococcus faecalis* strain 16 against which this antibiotic had no effect in broth. An optimal zone of bactericidal effect was shown in water but not in broth against *S. faecalis* strain 16 and the two strains of *Staphylococcus aureus* tested, but not against the two Gram-negative rods. For example, with

S. aureus strain Heatley, concentrations of 5–10 μg./ml. killed more rapidly in water than did either lower or higher amounts (Fig. 1).

Likewise, polymyxin was far more active in water than in nutrient broth in terms of either the bactericidal rate or of the minimal bactericidal dose. With streptomycin, the difference in the two environments was less marked, but the minimal dose was 5–25 times lower in water than in broth. No optimal zone was noted with either drug.

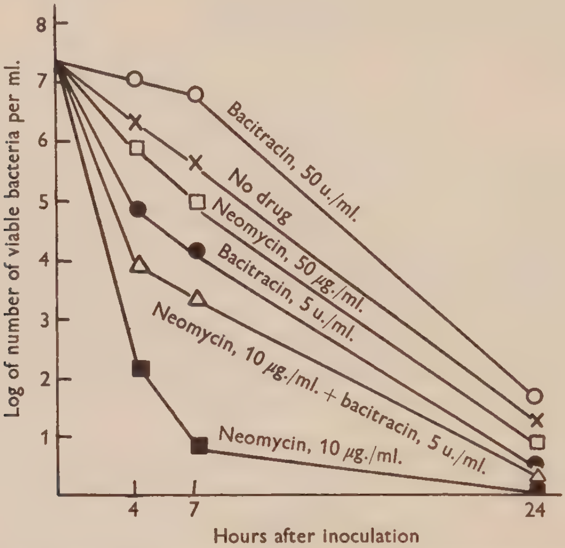

Fig. 1. **Bactericidal action of neomycin and bacitracin on *Staphylococcus aureus* strain Heatley suspended in distilled water at 37°, showing zone phenomenon. Neomycin is more rapidly bactericidal at a concentration of 10 μg./ml. than at 50 μg./ml.; bacitracin, at 5 units/ml. than at 50 units/ml. Bacitracin interferes with the bactericidal effect of neomycin. The organisms die more rapidly in the drug-free control than in bacitracin (50 units/ml.).**

Oxytetracycline showed approximately the same degree of bactericidal action on organisms suspended in either water or broth with a slight tendency toward a greater effect in water. There was no zone detectable.

Bacitracin did not differ sharply in its ability to kill bacteria in broth or in water, although it was slightly more active in the former. A zone effect was shown in water with the two strains of staphylococci as has been reported (Gunnison *et al.* 1954). With *Staphylococcus aureus* strain Heatley, the bactericidal rate of concentrations of bacitracin greater than the optimum was actually slower than that in water alone; i.e. the drug seemed to protect the organisms from the harmful effect of water and this may be responsible for the apparent zone (Fig. 1).

Penicillin, as expected, was far more effective in broth, although large doses (50–500 μg./ml.) had a bactericidal effect on some strains in water. As observed before a zone was shown with *Streptococcus faecalis* strain 16 only in broth (Gunnison, Jawetz & Coleman, 1950).

In summary, the six antibiotics showed a scale of activity in water as compared to that in broth, ranging from neomycin, polymyxin, and, to a less extent, streptomycin, which were more active in water; oxytetracycline and bacitracin which were almost equally active in water or broth; to penicillin which had little or no effect in water.

Table 2. *Effect of temperature on the bactericidal action of antibiotics in water and in broth*

	Minimum bactericidal dose* at											
	Neomycin		Polymyxin		Streptomycin		Oxytetracycline		Bacitracin		Penicillin	
Organism	37°	4°	37°	4°	37°	4°	37°	4°	37°	4°	37°	4°
	In water											
S. faecalis 16	0·05	>500	5	>500	20	>500	5	>500	10	>500	>500	>500
S. aureus Heatley	0·5	1	<5	>500	5	20	2	500	5	>500	500	>500
E. coli Da	0·25	0·5	2	<5	5	5	10	500	100	500	500	>500
	In broth											
S. faecalis 16	>500	>500	>500	>500	500	>500	20	>500	2	>500	5	>500
S. aureus Heatley	25	50	>500	>500	100	500	5	>500	2	>500	0·01	>500
E. coli Da	10	20	5	5	100	500	<500	>500	100	>100	<500	>500

* As defined p. 510. Penicillin and bacitracin expressed in units/ml.; other drugs in μg./ml.

In order to test the hypothesis that the effectiveness of the drugs was related to bacterial multiplication, tests were made at 4°, a temperature which inhibited detectable growth of the organisms (Table 2). At this low temperature, the bactericidal effect of the antibiotics varied widely with the microorganism, as shown in Table 2. For example, at 4° *Streptococcus faecalis* strain 16 was resistant to all six drugs tested (Fig. 2); whereas *Staphylococcus aureus* Heatley was killed by neomycin and by streptomycin in concentrations only slightly greater than those required at 37° in water. Hence, the greater effect at 37° of certain antibiotics in water than in nutrient media cannot be attributed solely to the non-multiplying resting state of the bacteria.

In a number of tests, bacteria which were not sensitive to a given antibiotic at 4° were first exposed to its action for 4–48 hr. at 37° and then refrigerated at 4° or vice versa. The bactericidal effect initiated at 37° was arrested promptly by refrigeration, and there was no further decrease in the number of viable organisms within 72 hr. at the lower temperature. On the other hand, with organisms exposed first at 4° the bacterial population remained unchanged for as long as 48 hr.; but upon placing at 37°, bactericidal action occurred (Fig. 2) sometimes more rapidly than with bacteria not previously refrigerated.

The effect of pH value is one of the many factors which must be considered, especially when comparing tests made in unbuffered water with those in broth buffered at pH 7·2. The aqueous solutions of antibiotic varied with concentration from pH 5·5 to 7·0 except for oxytetracycline which fell to pH 3·7–3·0 in concentrations of 50–500 μg./ml. All the antibiotics used were stable for the period of experiment in the range of pH tested. There was no consistent correlation between the variations in pH and the bactericidal action or the occurrence of the zone phenomenon.

Attempts to carry out tests in Ringer's solution were often unsuccessful because some of the test bacteria, especially the staphylococci, tended to die out rapidly in the absence of drug. Furthermore, both sodium and potassium salts interfered with the action of certain antibiotics, notably with that of neomycin (Gunnison *et al.* 1954). With those drugs and bacteria where Ringer's solution could be used, the results obtained were essentially the same as those in water. In imidazole buffer at pH 7·2, the test organisms survived in the drug-free control and the buffer did not interfere with drug action. The bactericidal action of the various antibiotics in this buffer was usually similar to that in water.

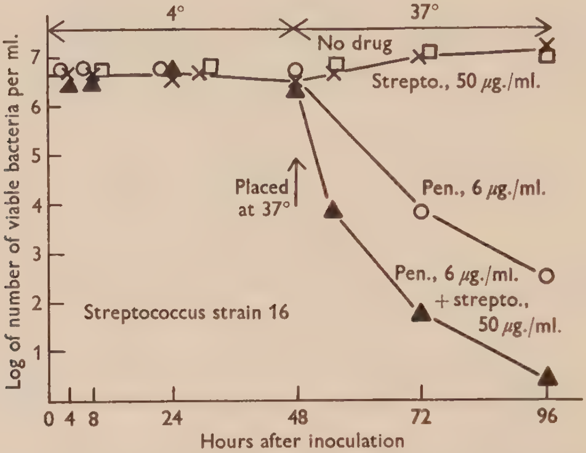

Fig. 2. Effect of temperature on bactericidal action of penicillin and streptomycin on *Streptococcus faecalis* strain 16 in broth. At 4°, these drugs have little effect alone or in combination. At 37°, the combination kills more rapidly than the optimal concentration of penicillin alone; i.e. synergism occurs.

Effects of pairs of antibiotics

Representative pairs of antibiotics known to show either synergism or antagonism when acting on certain bacteria in broth were tested for their combined effect on bacteria suspended in water at 37° with the results summarized in Table 3. The marked synergistic effects sometimes shown in broth were never demonstrable with non-multiplying bacteria in water. Mixtures such as penicillin + streptomycin, which were strongly synergistic when acting on *Streptococcus faecalis* strain 16 in nutrient medium, gave only addition or even indifference in water (Fig. 3). Even with those pairs where one or both drugs exerted a bactericidal action in non-nutrient solutions, synergism did not occur. For example, streptomycin + neomycin or oxytetracycline were synergistic against *Staphyococcus aureus* in broth but not in water, in spite of the ability of these drugs to kill the organisms in either environment.

Table 3. *Effect of combinations of antibiotics on bacteria exposed in nutrient broth and in water*

	S. faecalis 16		*S. aureus* H.		*S. aureus* De		*E. coli* Da	
Antibiotics tested	Broth	Water	Broth	Water	Broth	Water	Broth	Water
Penicillin								
+ streptomycin	Syn.	Add.	Syn.	Add.	0	0	0	—
+ neomycin	Syn.	Antag.	—	Add.	0	0	—	—
+ bacitracin	Syn.	0	Syn.	—	0	0	0	—
+ oxytetracycline	Antag.	0	Antag.	0	0	—	0	—
Streptomycin								
+ neomycin	0	Add.	Syn.	Add.	Syn.	Add.	0	Add.
+ bacitracin	Syn.	Add.	Syn.	0	Syn	0	0	0
+ oxytetracycline	0	Add.	Syn.	Antag.	Syn.	Antag.	0	0
+ polymyxin	—	Add.	—	—	—	—	Syn.	Add.
Neomycin								
+ bacitracin	Syn.	Add.	Syn.	Antag.	Syn.	Antag.	—	0
+ oxytetracycline	—	Add.	Syn.	Antag.	Syn.	—	0	Add.
+ polymyxin	—	—	—	—	—	—	—	0
Bacitracin								
+ oxytetracycline	—	Add.	Syn.	Antag.	Syn.	Antag.	0	0
Oxytetracycline								
+ polymyxin	—	Add.	—	—	—	—	Syn.	0

Syn. = synergism; Antag. = antagonism; Add. = addition; 0 = indifference; these terms are defined on p. 510. — = not tested.

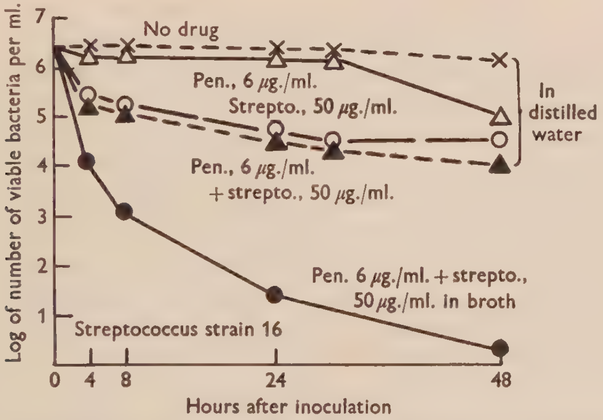

Fig. 3. Effect of medium on bactericidal action of penicillin and streptomycin on *Streptococcus faecalis* strain 16 at 37°. In distilled water, these drugs have little effect either alone or in combination. In broth, synergism occurs.

Mixtures which were antagonistic in broth, such as penicillin + oxytetracycline, were entirely indifferent in water. This was to be expected because, although inhibitory concentrations of oxytetracycline interfered with the bactericidal action of penicillin in broth, penicillin had no effect in water, so that it could not be antagonized. On the other hand, some pairs synergistic in broth appeared to be antagonistic in water. This was noted especially with bacitracin + oxytetracycline or neomycin and seemed due in part to the fact that the bacteria died more slowly in the presence of bacitracin than in the drug-free control (Fig. 1). Mixtures of streptomycin or neomycin + oxytetracycline in certain concentrations also were less effective together than alone against 'resting' staphylococci. In water and in buffer, penicillin interfered with the action of optimal amounts of neomycin on *Streptococcus faecalis* strain 16, although giving an additive effect with both lower and higher concentrations.

In a limited series of tests, bacterial growth was inhibited by holding at 4° in either broth or water. At this low temperature, no combined action other than occasional slight addition was observed even when one or both drugs showed a bactericidal effect when acting alone. With organisms first exposed to a pair of drugs at 37° and then refrigerated, the initial synergistic or antagonistic action was arrested. With organisms first exposed to two drugs at 4° and then transferred to the incubator, the usual sequence of events at 37° took place (Fig. 2). In tests made in Ringer's solution or in imidazole buffer the results were essentially the same as those in water. Thus it is unlikely that differences in pH were an important factor in these tests.

DISCUSSION

The bactericidal action of antibiotics in nutrient broth seems related in some degree to their effects *in vivo*, but their action in water or in buffer solutions probably reflects physical effects not operative *in vivo*. Hence, the latter experimental approach can give only limited answers concerning antibiotic action on non-multiplying bacteria in the body. An antibiotic might exert two effects on bacteria differing with concentration and conditions; one influencing their metabolism and the other altering their physical integrity.

The more rapid bactericidal action of neomycin and polymyxin in water than in broth may not be related to the resting state of the bacteria because when multiplication was suppressed by lowering the temperature in broth, the bactericidal rate was never increased and might be decreased. The action of these antibiotics in water or in buffer is probably not related to biosynthesis of actively metabolizing bacteria. Physical surface effects, disturbing osmotic equilibrium of the cell wall, have been demonstrated for polymyxin (Few & Schulman, 1953; Newton, 1954).

Although it has been established that penicillin is most effective against multiplying bacteria (Hobby, Meyer & Chaffee, 1942; Lee, Foley & Epstein, 1944), the situation with streptomycin is less clear. Garrod (1948) found that streptomycin had little effect at 5° in broth. Most workers have found streptomycin to be more effective against multiplying bacteria than against those in water or in salt solution (Hamre, Rake & Donovick, 1946; Hobby & Lenert, 1947; Strauss, 1947; Davis, 1954), a finding not confirmed here. This disagreement may be due partly to the strains tested.

Failure of streptomycin, neomycin, and polymyxin to kill some of the test bacteria rapidly in nutrient broth, although doing so in water, may be due in part to an interfering action of salts (Berkman, Henry & Housewright, 1947; Schoenhard & Stafseth, 1953; Newton, 1954). Although streptomycin and neomycin are known to increase in activity with pH value in nutrient broth (Waksman, Bugie & Schatz, 1944; Waksman & Lechevalier, 1949), there was no correlation between pH and bactericidal action in water.

Difficulties in interpreting the influence of various factors are increased when simultaneous action of two antibiotics is considered. Antagonism between two antibiotics had not been observed previously in the absence of multiplication (Jawetz & Gunnison, 1953). Although none of the pairs antagonistic in broth showed this effect in water, oddly some pairs synergistic in broth showed antagonism in a narrow range of concentrations in the absence of nutrients.

Inasmuch as synergistic action could not be demonstrated against non-multiplying bacteria, it seems unlikely that persisters are affected by combinations of drugs or that synergism in broth and *in vivo* is due to specific destruction of these members of a bacterial population. It seems more probable that in synergism in broth the bactericidal rate is so rapid that all exposed bacteria are killed before any of them enter into the state of reduced metabolic activity believed to be characteristic of the persister.

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The Influence of the Factors Determining Vi-type Specificity in *Salmonella typhi* on the Adaptation of Vi-phage II

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SUMMARY: All adaptations of Vi-phage II to the known Vi-types of *Salmonella typhi* have been grown on Vi-type A. In confirmation of earlier work these adaptations were found to be phenotypic or genotypic modifications of the original Vi-phage II, or showed a combination of both changes. Most adaptations to non-lysogenic types are changes of phenotype only, whereas those to lysogenic types are usually genotypically stable, although they may have a superimposed phenotypic modification. The fact that Vi-types that are exactly similar to naturally occurring types can be made artificially by the lysogenization of non-lysogenic types with type-determining phages makes it possible to assign structural formulae to most of the known naturally occurring Vi-types. The formulae consist of the symbol of the non-lysogenic precursor of a lysogenic type followed by that of the type-determining temperate phage in parenthesis. Thus, type F2 is type F1 carrying phage f2 and the formula of type F2 is therefore F1(f2). Using these formulae it is possible to predict the host range of Vi-phage II adapted to each lysogenic Vi-type. The phenotypic and genotypic changes concerned in the many adaptations of Vi-phage II are independent of one another, and by taking advantage of this independence it is possible to adduce experimental proof of the correctness of the proposed structural formulae for the lysogenically determined Vi-types. In studying the Vi-types it is useful to regroup those that are lysogenic according to the non-lysogenic precursor types common to certain of them.

The Vi-phage typing of *Salmonella typhi* (Craigie & Yen, 1938) is now recognized to be one of the most valuable guides to the epidemiology of typhoid fever. There are at present 33 recognized Vi-types of the typhoid bacillus. The convention, originated by Craigie and Yen, of designating Vi-types and their corresponding adapted phages by identical symbols is still maintained. The earlier types and their adapted phages were designated by capital letters, but most of those more recently identified have been assigned numerical symbols. The reactions of the internationally recognized Vi-types with the adapted phage preparations in routine test dilution* are shown in Table 1. As this Table shows, type A is sensitive to all the typing preparations, irrespective of their specificity.

The parent phage of the adapted typing preparations was named by Craigie & Yen (1938) Vi-phage II: it is specific for the Vi-positive form of the typhoid bacillus, and is not adsorbed by the Vi-negative form because of the absence of the Vi antigen which is necessary for its attachment to the bacterial cell. All

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* The routine test dilution of a phage is that giving confluent or semi-confluent lysis on the homologous type and a minimum of cross-reactions on heterologous types.

Vi-types of *Salmonella typhi* will adsorb all the adapted Vi-typing preparations, irrespective of their specificity (Craigie, 1940), but lysis only occurs when the type specificities of the organism and the phage coincide. A lethal effect on the bacteria is apparent when a culture of *S. typhi* adsorbs a heterologous Vi-typing preparation. This is especially marked when the phage multiplicity, that is, the phage/organism ratio, is high (Anderson & Fraser, unpublished). All the Vi-typing phage preparations are neutralized by anti-Vi-phage II serum prepared against phage A.

The factors which control Vi-type specificity in *Salmonella typhi* have been discussed in previous publications (Anderson, 1951; Felix & Anderson, 1951; Anderson & Felix, 1953*b*). It has been shown that Vi-type specificity in the typhoid bacillus is frequently an expression of a pattern of resistance of the organism to the typing phages which is dependent on lysogenicity of the particular types concerned. Of the 33 Vi-types recognized at present, 11 owe their specificity to this cause. The symbiotic or temperate phages responsible for Vi-type specificity are called type-determining phages. They are O phages and will attack and lysogenize non-Vi forms of the typhoid bacillus. In addition to controlling the reactions of strains of the typhoid bacillus with the Vi-typing phages, the type-determining phages also affect their sensitivity to O phages. Although the Vi antigen acts as the primary site of attachment of Vi-phage II to the typhoid bacillus, therefore, type specificity of which lysis by the adapted Vi-phages is an expression would appear to be determined elsewhere in the soma of the bacterial cell.

The type-determining phages are designated by small letters corresponding to the capitals by which the Vi-types concerned are known, and by a number with a superscript prime sign when the lysogenic Vi-type has a numerical designation. For example, type D1 carries phage d1, and type D6 phage d6; type 25 carries phage 25′, and type 26 phage 26′. Some types carry phages indistinguishable from those found in other types, in which case the phages are assigned an identical symbol. Thus, the determining phage of type 33 is indistinguishable from that of type D6 and the phage carried by both types is therefore called d6.

By lysogenizing selected non-lysogenic types with type-determining phages, Vi-types can be artificially prepared that appear to be identical with those occurring naturally. All but one (the exception is type D4) of the lysogenically determined types hitherto encountered in nature have now been prepared artificially in this way. In addition, several types not yet encountered in the field have been synthesized and it is possible to adapt Vi-phage II to most of these artificial new types to produce typing phages, usually of high specificity and reasonable working titre.

The alterations in host range that occur during the adaptation of Vi-phage II to the Vi-types of *Salmonella typhi* to produce the specific typing phages are in many cases caused by a change of phenotype only (‘host-induced modification’ of Luria & Human, 1952). The proof of the non-mutational character of this change lies in the results of propagation of the phages concerned on type A: every particle of a phenotypically modified preparation changes to phage A.

Table 1. *Reactions of Vi-type strains of* Salmonella typhi *with ro*

	Typing preparations of Vi-phag																
Vi-type strains	A	B1	B2	B3	C	D1	D2	D4	D5	D6	E1	E2	F1	F2	G	H	J
A	**CL**	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL
B1	–	**SCL**	**±**	**±**	–	–	–	±	–	+	–	–	±	+ + +	±	±	–
B2	SCL	**–**	**CL**	**+ +**	SCL	+ + ±	+ ±	+ + +	SCL	–	+ + +	+ +	+ + +	+	+ + +	+ + +	+ + ±
B3	SCL	**±**	**CL**	**CL**	CL	SCL	+ + ±	SCL	SCL	+ + +	SCL	CL	CL	SCL	SCL	SCL	SCL
C	±	±	±	±	**CL**	±	±	±	±	±	±	–	±	±	±	±	±
D1	–	–	–	–	–	**CL**	**SCL**	**CL**	**CL**	**SCL**	–	–	–	–	–	–	–
D2	–	–	–	–	–	**–**	**SCL**	**–**	**–**	**–**	–	–	–	–	–	–	–
D4	–	–	–	–	–	**–**	**–**	**CL**	**–**	**–**	–	–	–	–	–	–	–
D5	–	–	–	–	–	**±**	**–**	**–**	**CL**	**CL**	–	–	–	–	–	–	–
D6	–	–	–	–	–	**–**	**–**	**–**	**–**	**CL**	–	–	–	–	–	–	–
E1	–	±	–	±	–	–	–	±	±	–	**CL**	**CL**	–	±	–	–	–
E2	–	–	–	–	–	–	–	–	–	–	**–**	**CL**	–	–	–	–	–
F1	–	–	–	–	–	–	–	–	–	–	–	–	**CL**	**CL**	–	–	–
F2	–	–	–	–	–	–	–	–	–	–	–	–	**±**	**CL**	–	–	–
G	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**CL**	–	–
H	–	–	±	–	±	–	–	±	–	–	±	–	±	±	–	**CL**	±
J	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	**CL**
K	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L1	–	–	–	–	–	–	–	–	±	±	–	–	–	–	–	±	–
L2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
M	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
N	–	–	±	±	±	±	±	±	±	±	±	–	–	±	–	±	–
O	±	–	+ +	+ + +	+	–	–	–	–	–	±	–	±	+ + +	–	–	+
T	–	–	–	–	±	–	–	–	±	–	–	–	–	–	–	–	–
25 (Lie Kian Joe)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
26 (Clark)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
27 (Scholtens)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
28 (Scholtens)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
29 (Borman)	±	±	–	±	±	–	–	–	–	CL	–	–	±	CL	±	–	±
30 (Anderson)	–	–	–	–	±	–	–	–	–	±	–	–	–	±	–	–	–
31 (Wilson & Edwards)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
32 (Felix & Fraser)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
33 (Desranleau C_2)	–	–	–	–	–	–	–	–	–	±	–	–	–	–	–	–	–

CL = confluent lysis; SCL = semi-confluent lysis; + to + + + = increasing

Homologous and group reactions are shown in heavy type.

ne test dilutions of the typing phages

K	L1	L2	M	N	O	T	25	26	27	28	29	30	31	32	33	Vi-type strains
CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL	A
±	±	±	±	±	++	++	–	±	–	–	+±	++	–	–	++±	B 1
+±	++±	+++	±	SCL	+++	SCL	+++	SCL	+++	SCL	–	–	++	+++	–	B 2
SCL	CL	CL	SCL	CL	CL	CL	SCL	SCL	SCL	CL	++	+++	SCL	SCL	+	B 3
±	±	±	–	±	±	+	±	±	±	±	±	CL	±	–	CL	C
–	–	–	–	+	–	–	–	±	–	–	–	–	–	–	+++	D 1
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	D 2
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	D 4
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CL	D 5
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	CL	D 6
–	±	–	–	–	±	±	–	–	–	±	±	–	CL	–	±	E 1
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	E 2
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	F 1
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	F 2
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G
±	±	±	–	±	±	±	–	±	±	±	–	±	–	–	–	H
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	J
CL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	K
–	**CL**	**CL**	–	–	±	±	–	–	–	–	–	–	–	–	–	L 1
–	–	**CL**	–	–	–	–	–	–	–	–	–	–	–	–	–	L 2
–	–	–	**CL**	–	–	±	–	–	–	–	–	–	–	–	–	M
–	–	–	–	**CL**	–	±	–	±	±	++	–	–	–	–	–	N
–	±	±	+++	–	**CL**	±	–	–	–	–	+++	+++	±	–	–	O
–	±	–	–	–	–	**CL**	–	–	–	CL	–	–	–	–	–	T
–	–	–	–	–	–	–	**CL**	–	–	–	–	–	–	–	–	25 (Lie Kian Joe)
–	–	–	–	–	–	–	–	**CL**	–	–	–	–	SCL	–	–	26 (Clark)
–	–	–	–	–	–	–	–	–	**CL**	–	–	–	–	–	–	27 (Scholtens)
–	–	–	–	–	–	–	–	–	–	**SCL**	–	–	–	–	–	28 (Scholtens)
–	±	±	–	–	++	±	–	–	–	±	**CL**	CL	–	–	CL	29 (Borman)
–	–	–	–	–	–	–	–	–	–	–	±	**CL**	–	–	CL	30 (Anderson)
–	–	–	–	–	–	–	–	–	–	–	–	–	**CL**	–	–	31 (Wilson & Edwards)
–	–	–	–	–	–	–	–	–	–	–	–	–	–	**CL**	–	32 (Felix & Fraser)
–	–	–	–	–	–	–	–	–	–	–	–	+++	–	–	**CL**	33 (Desranleau C_2)

ers of discrete plaques; ± =less than 11 discrete plaques.

(*Facing p.* 520)

Some 'adapted' phages, however, are true host-range mutants and these may also undergo modifications of phenotype. Thus, the adapted Vi-typing preparations may owe their various host ranges to the following combinations: wild genotype, modified phenotype; modified genotype, wild phenotype; modified genotype, modified phenotype. Phage A, which has the narrowest host range of all the Vi-typing phages and seems to be the most primitive form of Vi-phage II, can be regarded as wild genotype, wild phenotype. Obviously, a host-range mutant descended from phage A, since it lyses other types than type A, is not of the same phenotype as phage A. However, as it has been found that changes in phenotype of the phage can occur without mutation, the convention has been adopted of referring to the preparations that have undergone this non-hereditary change as being phenotypically modified, and the phages that have undergone mutation only are described as being genotypically modified or as host-range mutants, with the implication that they retain the wild phenotype in the portion of the phage that is subject to host-induced modification. Host-range mutants that have undergone host-induced modification are described as phenotypically modified mutants.

Anderson & Felix (1952, 1953*a*, *c*) propagated a number of adapted Vi-typing preparations on type A and determined the host range of the phages so obtained. Ten phages were investigated: five changed to phage A, that is, they were phenotypic modifications as defined above; four were propagated unchanged (genotypic modifications or host-range mutants); and one changed its host range without becoming phage A (phenotypically modified mutant).

METHODS

Media. The nutrient basis of the media used in these experiments was Bacto dehydrated nutrient broth (Difco Laboratories). This was used in a strength of 2%, with the addition of 0·85% NaCl. The base for solid media was 1·3% New Zealand powdered agar. These media are referred to as 'Difco' broth and 'Difco' agar.

Titration of phages. Phages were titrated by the 'spot titration' method, serial dilutions being spotted with a standard loop of 3 mm. internal diameter on to a freshly spread lawn of organisms of the various Vi-types under test on Difco agar plates. These loops deliver a volume very close to 0·01 ml.

Preparation and identification of phages. The new phage preparations were grown as follows:

A lawn of logarithmically growing organisms of the type on which the phage was to be propagated was spread on the surface of Difco agar plates and allowed to dry. The phage under examination was applied to this culture in decimal dilutions. The plates were incubated overnight, and the following morning three to six discrete plaques, together with a small amount of surrounding culture, were cut from suitable dilutions. Each plaque was transferred to 20 ml. of Difco broth in a screw-capped bottle and incubated at 38·5° until no further lysis appeared to be taking place. The host cells were then killed by heating the bottles at 57° for 40 min., after which the lysates were centrifuged to remove the dead bacteria.

Phages were designated as fractions, the numerator indicating the starting phage, and the denominator the host strain on which it had grown. Thus, phage 26/E1 resulted from the growth of phage 26 on type E1. All the adapted phages described in the following pages were descended from single plaques. Three to six phage lines were grown in each experiment and most experiments were confirmed by repetition.

The type specificity of newly grown phages was identified by titrating them on a range of Vi-type strains. Type A, which is sensitive to all Vi-typing phages, was included in all titrations. The additional types used were: that on which the phage had formerly been grown to produce the specificity of which the stability was under examination; that on which the phage had most recently been grown; one or more related types if available that might be sensitive to the early or later adaptations; and a few types chosen at random that would be expected to be resistant to the former and the latest adaptations. For instance, phage 26/E1 was tested on types A, 26 (former adaptation), E1 (latest adaptation), 31 (related to types 26 and E1 (see Table 1) and expected to be lysed by the newly grown phage) and C and F1 (resistant to phages 26 and 31). Thus, by the use of a limited number of Vi-types, it was possible to establish the specificity of the new phages.

RESULTS

Table 2 shows the result of growing phage L2 on type A. This titration is given in full in order to indicate the range of Vi-types on which the phages were tested. A titration of phage L2 on the same range of types is given for purposes of comparison. The single-plaque phages in Table 2 had been cut from this titration of phage L2 on type A. It can be seen from Table 2 that phage L2, which normally attacks types A, L1 and L2 with equal readiness, had lost its ability to lyse the latter two types, had apparently not acquired new host ranges, and had retained only the capacity to attack type A with high efficiency. It had thus been converted into phage A.

Table 3 shows the result of growing all the adapted Vi-typing phages on type A. For the sake of completeness the ten typing phages examined by Anderson & Felix (1952, 1953*a*) are included. Phages B2 and B3 are omitted from Table 3 because their respective Vi-types have a low degree of specificity and show a wide range of sensitivity to many other adapted Vi-typing preparations (see Table 1). The specificity of phages B2 and B3 is difficult to distinguish from that of phage A, and satisfactory results could not be obtained in experiments carried out with them. Phage A is also omitted for obvious reasons.

The phages in Table 3 that had formerly been adapted to lysogenically determined Vi-types are starred. It can be seen that, of 13 phages showing the phenotypic change to phage A, 12 had been adapted to non-lysogenically determined types and one only (phage T) to a lysogenically determined type. On the other hand, 5 out of the 11 phages which were propagated unchanged had been grown on lysogenically determined types; and 5 of the 6 phages changing in specificity but not yielding phage A, being evidently phenotypic modifications of host-range mutants, had been propagated on lysogenically determined types. Thus, adaptation of Vi-phage II to non-lysogenically determined types appears to be mainly phenotypic in nature, whereas the changes in host range of this phage during propagation on lysogenically determined types are in most instances partly or wholly genotypic. The further examination of the types not yet shown to be under lysogenic control that elicit a genotypic change in Vi-phage II has not so far been carried out.

Table 2. *Propagation of phage L2 on type A*

			Phage L2/A. Preparations grown from single plaques		
Cultures	Phage dilutions	Phage L2	Plaque 1	Plaque 2	Plaque 3
Type A	10^{-4}	CL	CL	CL	CL
	10^{-5}	CL	<CL	<CL	<CL
	10^{-6}	<SCL	58	93	65
	10^{-7}	37	18	13	8
	10^{-8}	1	2	1	2
Type C	10^{-4}	7	1	—	—
	10^{-5}	—	—	—	—
	10^{-6}	—	—	—	—
	10^{-7}	—	—	—	—
	10^{-8}	—	—	—	—
Type E1	10^{-4}	—	—	—	—
	10^{-5}	—	—	—	—
	10^{-6}	—	—	—	—
	10^{-7}	—	—	—	—
	10^{-8}	—	—	—	—
Type F1	10^{-4}	—	—	—	—
	10^{-5}	—	—	—	—
	10^{-6}	—	—	—	—
	10^{-7}	—	—	—	—
	10^{-8}	—	—	—	—
Type J	10^{-4}	—	—	—	—
	10^{-5}	—	—	—	—
	10^{-6}	—	—	—	—
	10^{-7}	—	—	—	—
	10^{-8}	—	—	—	—
Type L1	10^{-4}	CL	2	—	—
	10^{-5}	CL	—	—	—
	10^{-6}	SCL	—	—	—
	10^{-7}	23	—	—	—
	10^{-8}	2	—	—	—
Type L2	10^{-4}	CL	1	3	1
	10^{-5}	SCL	—	—	—
	10^{-6}	+++	—	—	—
	10^{-7}	35	—	—	—
	10^{-8}	3	—	—	—

CL = confluent lysis; SCL = semi-confluent lysis; <CL and <SCL = intermediate degrees of lysis; +++ = more than 150 plaques; 1, 2, 3, etc. = number of discrete plaques.

Attention was concentrated on the phages that had undergone a change suggesting that they were phenotypic modifications of host-range mutants.

These are shown in the third column of Table 3. They are listed separately in Table 4, together with the change in specificity they sustained after passage on type A, and the determining phages carried by the types on which they had originally been grown. As phage D2 had been adapted to an apparently non-lysogenic type, it is not considered in the succeeding discussion.

Table 3. *Results of propagation of specific Vi-typing phages on type A*

Phages which changed to phage A (phenotypic change only)	Phages propagated unchanged (host-range mutants)	Phages undergoing a change but not to phage A (phenotypically modified mutants)
C†	B1	D2
E1†	D1*	D4*†
E2	D5†	F2*
F1†	D6*†	30*
G	K‡	31*
H	N†	33*
J	O†	—
L1†	25*	—
L2	26*	—
M	28‡	—
T*†	29*	—
27	—	—
32	—	—

* Phages corresponding to lysogenically determined types.

† Previously examined by Anderson & Felix (1952, 1953*a*).

‡ Types K and 28 are lysogenic but their temperate phages do not appear to be concerned with type determination.

Table 4. *Vi-typing phages undergoing modification when grown on type A but not reverting to phage A*

Starting phage	Resulting phage	Determining phage carried by Vi-type on which starting phage has been grown
(1)	(2)	(3)
D2	D1	—
D4	D1	d1
F2	29	f2
30	29	f2
31	26	26′
33	D6	d6

The specificities of the phages in column 2 of Table 4 that had been originally adapted to lysogenic Vi-types are exactly those that would result from the growth of phage A on types D1, 29, 26 and D6 respectively. These types can be prepared from type A by lysogenizing it with phages d1, f2, 26′ and d6. We may now consider the types corresponding to the phages in column 1 of Table 4. The non-lysogenic type from which type D4 can be prepared is not known, but type F2 can be prepared from type F1 by lysogenizing it with phage f2; type 30 from type C with phage f2; type 31 from type E1 with phage 26′; and type 33 from type C with phage d6. Reference to Table 3 will show that the phages adapted to types F1, C and E1 are phenotypically modified, and all change back to phage A when grown on type A. The same change had manifestly occurred in phages F2, 30, 31 and 33, but reversion of their host ranges to that of phage A was impossible because each adapted phage had already undergone the genotypic changes necessary to overcome the resistance conferred on the original host cells by their temperate determining phages. Thus, the end result of the growth of the Vi phages in column 1 of Table 4 on type A was to produce phages with specificities suggesting that they had been grown on a series of type A cultures lysogenized with type-determining phages f2, 26′ and d6. Although the non-lysogenic precursor of type D4 is unknown, the change in phage D4 was clearly of the same nature. It is evident, therefore, that the effect of growth in type A cells on the specificity of the phages in column 1 was restricted to that portion of the phages that had been modified by the non-lysogenic component of each lysogenic complex on which they had originally been grown.

The structural formulae of the Vi-types

The information provided by the fact that naturally occurring Vi-types can be replicated in the laboratory by lysogenizing non-lysogenic types with suitable type-determining phages enables the lysogenically determined types to be considered on a structural basis. Adopting the current convention of designating a lysogenic strain by the symbol of the host cell followed by that of the carried phage in parenthesis, a series of structural formulae have been evolved for the Vi-types that owe their specificity at least in part to lysogenesis. The use of such formulae was first suggested by Luria (1953). The formulae used by the present writers are shown in Table 5; these give information con-

Table 5. *The structural formulae of the lysogenically determined Vi-types of* Salmonella typhi

Original designation of Vi-type (1)	Type-determining phage carried (2)	Structural formula of Vi-type (3)	Types fully lysed by homologous adaptation of Vi-phage II in routine test dilution (4)
D1	d1	A(d1)	A, D1
D4	d1	?(d1)*	A, D1, D4
D6	d6	A(d6)†	A, D6, 29
F2	f2	F1(f2)	A, F1, F2, 29
T	t	A(t)	A, T
25 (Lie Kian Joe)	25′	A(25′)	A, 25
26 (Clark)	26′	A (26′)	A, 26
29 (Borman)	f2 or 30′	A(f2) or A(30′)†	A, 29
30 (Anderson)	f2 or 30′	C(f2) or C(30′)†	A, C, 29, 30
31 (Wilson & Edwards)	26′	E1(26′)	A, E1, 26, 31
33 (Desranleau C2)	d6	C(d6)	A, C, D6, 29, 30, 33

* The non-lysogenic precursor of type D4 is at present unknown.

† Phages d6, f2 and 30′ belong to a single serological group; they are also closely related in type-determining properties.

cerning the phylogenetic relationships of the Vi-types concerned and also make it possible to predict the host ranges of the Vi-phage II preparations adapted to them. For example, type D6 is structurally A(d6) and type 33 is C(d6). When Vi-phage II is adapted to C(d6) it acquires simultaneously the lytic range of phage C and that of phage D6. Such an adaptation, therefore, will lyse type A (which is sensitive to all Vi-phage II adaptations), type C, and also all lysogenic types descended from these two non-lysogenic precursors by lysogenization with phage d6 or a phage closely related to it. Phages f2 and 30′ are closely related to phage d6. Thus, the additional types lysed by phage 33 are: D6(=A(d6)); 29(=A(f2) or A(30′)); 30(=C(f2) or C(30′)); and 33(=C(d6)) on which the phage was propagated. It must be pointed out here that, although phages f2 and 30′ do not confer on a strain the property of resistance to phage D6, carriage of phage d6 endows cells with relative or complete resistance to Vi-phage II adapted to strains carrying phages f2 or 30′. Indeed, this resistance is the only known distinguishing feature between phages d6 and f2. Strains lysogenized with phages f2 and 30′ show identical resistance patterns to the Vi-phage II typing preparations. They also select the same host-range mutant of Vi-phage II when the latter is grown on them. Phage f2 is distinct from phage 30′, however, because the former makes the strains it lysogenizes resistant to Craigie and Yen's Vi-phage IV whereas the latter does not. The determining phage of type 29 was formerly designated as phage 29′ (Anderson & Felix, 1953*b*). Later work, however, has shown that this phage is indistinguishable from phage f2 and it is no longer considered by the present writers to merit separate recognition.

By a similar approach to that outlined for phage 33, the host range of phage 31 can be predicted. Type 31 is E1(26′). Vi-phage II adapted to it has the lytic range of phage E1 plus that of phage 26. The Vi-types lysed by phage 31 are, therefore: type A; type E1; and any lysogenic types carrying phage 26′ that are descended from these two precursors. Such types are type 26(=A(26′)) and type 31(=E1(26′)) on which the phage was grown.

A lysogenically determined type is not sensitive to Vi-phage II adapted to another type carrying the same or a similar type-determining phage unless the non-lysogenic component of the first type is the same as that of the second or is type A, which is sensitive to all adaptations of Vi-phage II. Thus, type F2, which is structurally F1(f2), is resistant to phages 29 and 30, both adapted to types carrying phage f2 or a phage very closely related to it (see Table 4). Phages F2, 29 and 30 all exhibit the same permanent genotypic change resulting from their being grown on strains carrying phage f2. In considering the relations of the specificities of these phages to one another, therefore, this permanent alteration can be disregarded and the differences between phages F2, 29 and 30 become identical with those between adaptations of Vi-phage II to types F1, A and C, which are the non-lysogenic precursors of types F2(=F1(f2)), 29(=A(f2)) and 30(=C(f2)) respectively. Neither phage A nor phage C will lyse type F1; therefore, neither phage 29 nor phage 30 will lyse type F2.

The host ranges of the adapted phages shown in column 4 of Table 5 have been restricted to parental non-lysogenic types and types carrying phages indistinguishable from or closely related to the phage determining the Vi-type to which Vi-phage II has been adapted. There are, however, other types, some lysogenic, others non-lysogenic, that are almost as sensitive to some heterologous adapted phages as they are to their homologous typing preparations. Types D1 and D5 are examples of this phenomenon. Both of these types are sensitive to phage D6. This is not shown in Table 5 but is in Table 1. In the case of type D1, this sensitivity can be otherwise stated as indicating that lysogenization of type A with phage d1 will not equip the cell with resistance against the host-range mutant of Vi-phage II that can lyse type D6. The reason for the phage sensitivity pattern of type D5, which seems to be non-lysogenic, is not yet known, but its sensitivity to phage D6 is quite clear-cut. If a culture is sensitive to phage D6, it will be sensitive to adaptations of Vi-phage II to any other types carrying phage d6. This is because type D6 is structurally A(d6) and in adaptation to this type Vi-phage II undergoes two changes: a mutation that enables it to overcome the block in its multiplication produced by the presence of phage d6 in the cell; and a phenotypic conditioning identical with that of phage A which has the narrowest of all host ranges. Type 33 is C(d6): adaptation of Vi-phage II to it demands the same host-range mutation as that present in phage D6 and a phenotypic modification identical with that of phage C. The mutation common to phages D6 and 33 can be ignored when comparing their host ranges. The difference between the two phages then becomes identical with that between phage A and phage C. Any strain attacked by phage A will be sensitive to phage C; therefore, any strain lysed by phage D6 should be lysed by phage 33. This hypothesis is supported by the fact that types D1 and D5, which are sensitive to phage D6, are also sensitive to phage 33. Applying the same arguments, types D1 and D5 should be sensitive to Vi-phage II adapted to any other type carrying phage d6, irrespective of the non-lysogenic precursor of the type. An unnamed new type, identified in this laboratory in 1952, is at present known as T4904; it has the structural formula E1(d6). Phage A could not be adapted to this type, but phage D6 grew on it with ease to produce a phage which attacked types A, E1, D6 and T4904 with equal facility. As shown above, this phage should also be able to lyse types D1 and D5 and it does so.

The independence of the phenotypic and genotypic changes in Vi-phage II

A study of the formulae of the lysogenically determined types suggested that the adaptation of Vi-phage II to them consisted of the two separate processes of phenotypic modification and genotypic change, and it was decided to examine the extent to which these were independent of one another. Phages adapted to a selection of the lysogenically determined types were grown on non-lysogenic types known to be the precursors of other lysogenically determined complexes. Strain T4904(=E1(d6)), referred to earlier, was included in these experiments. Two types prepared artificially in the laboratory were also included. One of these was E1(f2) which was prepared by one of us (E.S.A.) in 1951. This has been referred to previously as 'a new subtype of the E group' (Felix & Anderson, 1951; Anderson & Felix, 1953*b*). Since these experiments were performed this type has been discovered in the field by Dr R. T. Scholtens (1955). We have examined the culture discovered by Scholtens, and find its formula to be E1(30′). As has been indicated (see Table 5), types carrying phages f2 or 30′ present an identical lytic pattern with the adaptations of Vi-phage II. This type has been provisionally designated type E7. The second artificially prepared type used in these experiments was E1(d1) which is a further subtype of the E group.

In each case the non-lysogenic Vi-type chosen as the new host was known to be the precursor of a lysogenically determined type carrying a phage indistinguishable from that carried by the type on which the starting phage had originally been grown. For example, phage F2 was originally adapted to type F2, which is structurally F1(f2). Phage F2 was grown on type A, which is the non-lysogenic precursor of Type 29(=A(f2)). Since the change in Vi-phage II that enables it to overcome the resistance conferred on the cell by the presence of phage f2 is genotypic in nature and therefore stable, it was expected that the only change that would occur in phage F2 as the result of growth on type A would be that affecting the phenotypically controlled F1 specificity which should change to that of phage A. Thus, the specificity of phage F2 should change under these conditions from that corresponding to the F1(f2) complex to that corresponding to the A(f2) complex and the resulting phage should therefore be identical with phage 29 prepared by growing phage A on type 29. This prediction was confirmed and the principle was successfully applied to a number of other Vi-phage II typing preparations in which both phenotypic and genotypic changes were known to be present. These experiments are summarized in Table 6. The host ranges of the new phages shown in the fourth column of Table 6 were found to be identical with those of Vi-phage II preparations grown on the lysogenic types listed in the last column. All these host ranges had been predicted before the respective experiments were carried out.

These findings show that the phenotypic and genotypic changes in Vi-phage II are two entirely independent processes. Each host-range mutant as far as it was tested showed a phenotypic plasticity similar to that of phage A. The experimental results also prove the validity of the suggested structural formulae for the lysogenically determined Vi-types.

DISCUSSION

It was shown in earlier work (Anderson & Felix, 1952, 1953*a*, *c*) that the changes in host range that occur in Vi-phage II when it is adapted to the various Vi-types of *Salmonella typhi* could be either phenotypic or genotypic in nature. In one instance, that of phage D4, both changes appeared to be represented. These observations were carried out on a limited number of Vi-typing preparations. The exploration of the complete range of Vi-typing phages has yielded the interesting result that most of the phenotypically changed preparations have been adapted to non-lysogenically determined Vi-types, whereas adaptation of Vi-phage II to lysogenically determined types usually requires genotypic or both genotypic and phenotypic changes. It is quite clear from the present series of experiments that the two alternative types of modification are independent of each other: it seems probable that they take place at different sites in Vi-phage II and concern different steps in the multiplication cycle of the phage.

Table 6. *The adaptation to non-lysogenic types of Vi-typing phages originally grown on lysogenic types*

Starting phage	Structural formula of lysogenic type on which starting phage was grown	Non-lysogenic type on which starting phage is propagated	Type specificity of resulting phage	Structural formula of type to which new phage corresponds
D6	A(d6)	C	33	C(d6) = type 33
D6	A(d6)	E1	4904	E1(d6) = T4904*
33	C(d6)	A	D6	A(d6) = type D6
33	C(d6)	E1	4904	E1(d6) = T4904
F2	F1(f2)	A	29	A(f2) = type 29
F2	F1(f2)	C	30	C(f2) = type 30
29	A(f2)	C	30	C(f2) = type 30
30	C(f2)	A	29	A(f2) = type 29
26	A(26′)	E1	31	E1(26′) = type 31
31	E1(26′)	A	26	A(26′) = type 26
D1	A(d1)	E1	E1(d1)	E1(d1)†
F2	F1(f2)	E1	E1(f2)	E1(f2)†

* See reference in text. † Artificially prepared types.

Phenotypic modification affecting phages was first reported by Luria & Human (1952) in phage T2. Bertani & Weigle (1953) and Weigle & Bertani (1953) studied phenotypic changes in phage P2, a temperate phage isolated from *Escherichia coli* by Lisbonne & Carrère, and λ, the temperate phage carried by *E. coli* K-12. Fluctuation tests carried out by the latter workers with single cycles of phage multiplication showed that the number of plaques produced by a phage on a new culture, to which it could be phenotypically adapted but on which only a small fraction of the viable particles would initially form plaques, conformed to a Poisson distribution. From this they concluded that the particles undergoing adaptation to the new host were not produced by spontaneous mutation in the original stock of phage. Other experiments performed by these workers suggested that the potentiality to produce the altered host range in the phage was a property of exceptional cells of the new host and not of exceptional particles of the phage. The subject of phenotypic (or 'host induced') modification in phage has been reviewed by Luria (1953).

For the unequivocal demonstration that a change of host range in phage is purely phenotypic in nature it is necessary to show that it is completed in a single cycle of phage growth in the host cells. The work reported here, although performed in all cases with single-plaque lines of phage, was not checked by single-cycle experiments. Nevertheless, because of the uniformity of host range of all phage lines descended from single plaques picked from the growth on type A of any typing phage which has been designated as a phenotypic or partly phenotypic modification of Vi-phage II, it seemed reasonable to regard such preparations as having undergone a phenotypic change only. We have recently carried out single-cycle experiments which justify this assumption and similar observations have been made by Baron, Formal & Spilman (1955). We have also shown that the genotypically changed preparations are descended from selected host-range mutants that occur in concentrated preparations of phage A and that the mutations are independent of the presence of the heterologous bacterial types. An account of this work will be given in a later publication.

The use of the structural formulae proposed for the lysogenically determined types offers considerable advantages over the single-symbol designations in current use in the study of the factors controlling Vi-type specificity. As we have pointed out earlier, these formulae show the phylogenetic relationships of the lysogenic types. These types can be regarded as consisting of two separate components: one is the non-lysogenic ancestor of the type, which produces a phenotypic change only in Vi-phage II; and the other is the temperate phage which, in virtue of its association with the nuclear apparatus of the cell, is able to effect a block in the multiplication of the wild genotype of Vi-phage II. This block can only be by-passed or otherwise overcome by host-range mutants of the Vi phage. The site or factor in the cell which controls the phenotypic modification of the Vi phage is independent of, and unaffected by, lysogenization by type-determining phages. It can be regarded as a permanent characteristic of the cell that may be masked but not changed by the attainment of the lysogenic state. An apparent exception to this rule is found in type T, which is structurally A(t) (see Table 5): in this case the determining phage seems to induce only a phenotypic change in Vi-phage II.

The structural formulae show what phenotypic modifications are superimposed on particular mutants in the preparation of some of the Vi-typing phages and make it possible to predict the host ranges of the respective adaptations. Moreover, the formulae indicate the host-range mutant that can be grown on a lysogenically determined type if a typing phage of reasonable titre is difficult or impossible to prepare by propagating phage A on it. For example, it is rather difficult to grow phage A on type 33(= C(d 6); see Table 5) so as to produce a high-titre typing preparation in a single manipulation. On the other hand, phage D6 (a host-range mutant) will grow quite easily on type 33 to produce high-titre typing preparations which are indistinguishable in specificity from those prepared by the more conventional procedure. In fact, as has been shown (see Table 6) a typing phage of identical specificity can be prepared by growing phage D6 on type C.

It is the present custom to group Vi-types solely according to their reactions with the adapted typing preparations. The lysogenically determined types, however, could be grouped according to the non-lysogenic types from which they are derived and the phages they carry. As these two factors control the reactions of a strain with the typing phages, they each present a basis for classification. In Table 7 the types concerned are grouped on the basis of common non-lysogenic ancestry, the 'parent' type being placed at the head of each group. A number of non-lysogenic types exist that are related to type E 1 in a way that is at present obscure. These types are mostly sensitive only to their adapted Vi-phages but these phages will also readily lyse type E 1. Type E 2 is an example of this phenomenon (see Table 1); and other instances have been discovered by various workers. These types are omitted from Table 7, which is concerned only with the relations of non-lysogenic precursors to lysogenically determined types of which the structural formulae are known. Type D 4, which carries phage d 1, cannot at present be inserted into this scheme because its non-lysogenic precursor is unknown. New lysogenically determined Vi-types can be added to the scheme shown in Table 7 as they are discovered.

Table 7. *A classification of Vi-types of* Salmonella typhi by *their structural formulae*

Group	Original designation	Formula
A	A	A
	D1	A(d1)
	D6	A(d6)*
	29 (Borman)	A(f2) or A(30′)*
	T	A(t)
	25 (Lie Kian Joe)	A(25′)
	26 (Clark)	A(26′)
C	C	C
	30 (Anderson)	C(f2) or C(30′)
	33 (Desranleau C2)	C(d6)
E	E1	E1
	T4904	E1(d6)
	31 (Wilson & Edwards)	E1(26′)
	Artificially prepared types	E1(d1) E1(f2)
F	F1	F1
	F2	F1(f2)

* See footnote to Table 5 concerning phages d6, f2 and 30′.

It would, of course, be equally possible to classify lysogenic strains according to the phages they carry, but subdivision on this basis would necessarily omit related non-lysogenic types and full use would not be made of the available information. The proposed nomenclature and supplementary method of classification clarify the interactions of the Vi-phage types and their respective typing phages. As we have stated earlier, experiments have been carried out to examine the statistical distribution of particles undergoing phenotypic and genotypic changes. These form the subject of a separate communication which will be published in the near future.

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The Stability of *Lactobacillus* Strains

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SUMMARY: Reported changes of pure strains of *Lactobacillus* from the heterofermentative to the homofermentative type, with concomitant changes in many characters of recognized taxonomic importance, were investigated. No evidence was obtained that such gross variations occur. Inadequate initial purification and subsequent contamination between strains of different physiological types, were found to produce effects which could be mistaken for such changes.

The literature contains many allusions to the morphological and physiological variability of *Lactobacillus* in artificial culture. Most of these reports concern minor variations in, for instance, fermentation reactions (Rosebury, Linton & Buchbinder, 1929; McIntosh, James & Lazarus-Barlow, 1922, 1924). In recent years such variations have been recognized as of little taxonomic importance and do not impede the accurate classification of lactobacilli.

Although Cannon (1924) stated that no appreciable variation in fermentation reaction powers occurred as a result of prolonged culture of lactobacilli, Howitt (1930) regarded prolonged culture as a necessary stabilizing process, without which reliable classification could not be attained. Harrison (1942*a*, *b*) reported a type of variation in the physiological characters of oral *Lactobacillus* strains during the course of prolonged artificial culture which appeared to support Howitt's theory and was clearly of major taxonomic importance. Harrison (1942*a*) classified oral lactobacilli into two main types, groups A and B, on the basis of mannitol and sorbitol fermentation and acid production in milk. In the light of modern studies it is almost certain that group A represented the homofermentative *L. casei* and group B, the heterofermentative *L. fermenti* (Davis, 1955). Harrison (1942*b*) claimed that group B strains (mannitol- and sorbitol-negative) tended to acquire the morphological, physiological and serological characteristics of group A when maintained for some time in artificial culture. Although this apparent change from the group B to the group A type was observed in many strains, Harrison only found one group A strain which changed to the group B type. Possible explanations of these observations offered by Harrison were the acquisition of protoplasmic characters, development of adaptive enzymes, selection of variant cells or colonies, or the initial presence of mixed cell populations in apparently pure cultures.

Harrison's work was amplified by Coolidge, Williams, Ebisch & Hodges (1949), Coolidge (1950), Hodges, Coolidge & Harrison (1951), and Coolidge (1951) who claimed that the changes observed in physiology, serology, morphology and nutrition, in certain strains, were in fact the result of changes from the heterofermentative to the homofermentative type. The disappearance of the characteristically heterofermentative requirement for thiamin, and changes in the end products of glucose fermentation were recorded for heterofermentative cultures, derived from single cell isolates, in which metabolic changes were considered to have occurred. Nutritional adaptation during growth upon thiamin-free media was not found to produce this change; it appeared to occur very readily, however, in strains subjected to washing in 0·85 % saline solution. Washing in other metallic salt solutions did not appear to affect the rate of change. These studies gave the impression that the gross metabolic changes reported were caused by some process resembling a genetic mutation of the entire cell population in the cultures examined. In view of the taxonomic implications of such a process, if it should indeed prove to occur, the present investigations were made.

METHODS

The strains employed were of oral origin, maintained and classified by the methods previously described (Davis, 1955; Davis, Bisset & Hale, 1955). The techniques used were essentially the same as those of Coolidge and co-workers, but it should be noted that the medium employed, tomato juice (TJ) broth and agar, and the methods of strain characterization were somewhat improved as a result of recent studies in this field (see Davis, 1955, for literature). In all, 7 strains of *Lactobacillus casei* and 9 of *L. fermenti* were employed in the following experiments as homofermentative and heterofermentative representatives of the genus respectively.

EXPERIMENTAL

Experiment 1. *Effect of washing cells in* 0·85 % *sodium chloride.* Three strains of *Lactobacillus casei* and two of *L. fermenti* were checked for purity by repeating three times the selection of discrete colonies from 48 hr. surface cultures upon TJ agar. These were then inoculated from 24 hr. TJ broth cultures using capillary pipettes, to gas production and fermentation reaction media (Davis, 1955) to check the strain characteristics, and into duplicate TJ broths which were incubated for 24 hr. at 37°. One broth culture was then used to inoculate fermentation tests and TJ broth direct; the other was washed three times in 0·85 % sodium chloride solution and used to inoculate fermentation tests and TJ broth. The total period of time for which the cells of any one subculture were in contact with saline solution was never more than 50 min., during washing and resuspension. This was repeated every 48 hr. for 20 days, giving two parallel series of cultures for each strain, one washed in saline between subcultures and the other acting as an unwashed control series. The strain characters, as shown by fermentation reaction patterns, were recorded for each subculture of the washed and control series.

Results. The original fermentation patterns (subculture no. 1) of the five strains are shown in Table 1. During the course of ten successive transfers in TJ broth without washing in saline, no variation in the fermentation patterns of homofermentative strains (81, 5, 99) occurred. Of the heterofermentative strains, no. 68 fermented mannitol weakly (colour change required 12 days to become apparent) in the 6th subculture but not in subsequent ones. Strain 119 fermented salicin in the 3rd and 4th subcultures only. At the end of the experiment the fermentation patterns of all strains were exactly the same as at the beginning.

Table 1. *Original fermentation patterns*

Strain	Raffinose	Xylose	Lactose	Arabinose	Salicin	Rhamnose	Mannitol	Sorbitol	Species
81	−	−	+	−	+	−	+	+	*L. casei*
5	−	−	+	−	+	+	+	+	*L. casei*
99	−	−	+	−	+	−	+	−	*L. casei*
68	+	−	+	−	−	−	−	−	*L. fermenti*
119	+	−	+	−	−	−	−	−	*L. fermenti*

All fermentations apparent within 6 days.

In the series which underwent saline washing, homofermentative strains showed no variation from the original fermentation patterns. Strain 68 fermented sorbitol and mannitol weakly in the first suspension, but subsequent suspensions were inactive toward these sugars. Strain 119, however, showed definite and permanent changes in its fermentation patterns as the experiment proceeded. From the first saline suspension, mannitol and sorbitol were fermented; from the second, salicin, and from the ninth, rhamnose were also fermented. The only sugars not fermented by the tenth suspension were therefore xylose and arabinose. The cumulative effect was indicated by an ability to ferment greater numbers of the sugars tested, and an increased rate of fermentations in later suspensions. For instance, the second suspension required 14 days to ferment sorbitol, whereas the tenth suspension fermented sorbitol in only 4 days.

Superficially this result resembles those of Harrison and his co-workers. The fermentation of mannitol and sorbitol by strains originally incapable of fermenting these sugars was the main criterion of change used by Harrison (1942*a*, *b*). The occurrence of a marked and permanent change in a heterofermentative strain only, and that only when washed in saline, also appeared to confirm the claims of Coolidge. However, when at the end of the experiment, the final cultures of strain 119, derived from both the washed and control series were again tested for gas production, both were positive. The washed culture was thus shown to have remained heterofermentative in character. It was also observed that although the washed culture had acquired the fermentation characters of *Lactobacillus casei*, it had not lost those of *L. fermenti*. These facts suggested that the washed culture contained lactobacilli of both the *L. casei* and *L. fermenti* type. This was checked by culturing the final washed and unwashed cultures of strain 119 upon TJ agar, selecting six representative colonies from each and examining their characteristics. The results are shown in Table 2.

The ready separation of the two components (1 and 2) of the washed culture, showed conclusively that the aberrant fermentation reactions recorded during the course of the experiment were caused by the occurrence of a mixed cell population containing lactobacilli of both the *Lactobacillus casei* and *L. fermenti* type.

This experiment was repeated using 3 strains of *Lactobacillus casei* and 5 of *L. fermenti* not examined in the first experiment. 4 of the *L. fermenti* strains were found to contain cells of the *L. casei* type after only one subculture and washing in saline. Unwashed subcultures remained pure through ten transfers in TJ broth.

Table 2

Strain 119	Colony	Cell form	Gas	Fermentations
Unwashed	Large, semi-rough	Single and paired short bacilli	+	Raffinose and lactose
Washed	(1) Large semi-rough	Single and paired short bacilli	+	Raffinose and lactose
	(2) Small smooth	Bacilli in chains	–	Lactose, rhamnose, salicin, mannitol and sorbitol

It is clear that the most probable explanation of these results was simple contamination of *Lactobacillus fermenti* cultures by the *L. casei* cultures handled in the same experiment. The accurate designation of strains to certain species now possible in this genus, makes the explanation of the mixed cultures, as being due to contamination, much more easily discernible than it is if the strains are only known as heterofermentative or homofermentative, which was in fact the only designation that Coolidge and his colleagues could give to their strains. The experiments of Coolidge involved the handling of homofermentative and heterofermentative strains at the same time, as in Expt. 1 above, and contamination could therefore have occurred. It is certain that the process of centrifuging, decanting and resuspending cultures, although conducted with normal sterile precautions, is more open to contamination than is that of straightforward subculturing. Coolidge *et al.* (1949) state: 'The coincidence of changes in the two organisms (two heterofermentative strains) on the same day after repeated failure to change, suggests an uncontrolled factor in the medium, technique or environment'; and later, Hodges *et al.* (1951) write: 'Some factor associated with the preparation of washed cell suspensions of the organisms increases the incidence of the change'. In our opinion, contamination was the unknown factor.

Experiment 2. At different times and by different operators, 2 strains of *Lactobacillus fermenti* were separately treated as in Expt. 1. Both strains were from stock cultures of which previous subcultures had become mixed when used in Expt. 1. They were examined alone, i.e. no homofermentative or other heterofermentative strains were handled at the same time.

Result. In neither case did any change occur in the fermentation patterns of washed or unwashed subcultures, and at the end of the experiments both strains were pure and had their original characters. This showed that in the absence of possible contaminants, the strains were not affected by the treatment administered to them, and was considered to give support to the present contamination theory. It is possible that the mixed cell populations detected in

Expt. 1 might arise from the initial presence, in apparently pure cultures, of undetected minorities of alien cells which, under the action of selective forces, increased to detectable proportions.

Experiment 3. The question of initial impurity was satisfactorily answered by using cultures derived from single cells isolated with the aid of the De Fonbrune micromanipulator. One strain of *Lactobacillus casei* and one of *L. fermenti* were thus purified and examined together, as in Expt. 1.

Result. In the series of cultures subjected to washing, mixing of the two strains occurred quite rapidly, the presence of cells of the *Lactobacillus casei* type in the cultures of *L. fermenti* was the most easy to detect, but contamination of the *L. casei* cultures by cells of the *L. fermenti* type was also found to occur. No changes occurred in the unwashed control cultures.

Subsidiary experiments were also made to determine the possible selective action of sodium chloride at various concentrations and over various periods of time upon *Lactobacillus* strains of the two species being tested. The viable count method of Miles & Misra (1938) was used, as well as qualitative tests by cultural methods to determine their survival in, and tolerance of sodium chloride. The results showed that no great distinction existed between *L. casei* and *L. fermenti* in this respect, and it was therefore unlikely to be a factor in the selection of cells of either type in initially impure cultures. It is certain, however, that in most media used for Lactobacillus cultivation, cells of the casei type grow more rapidly and luxuriantly at 37°, than do those of other species. In consequence an initial, undetectable minority of such cells may increase in proportion in succeeding subcultures and even completely overgrow the culture. This is undoubtedly the manner in which the 'stabilizing effect' of prolonged culture (Howitt, 1930) was produced. The contemporary use of media more adequately designed to meet the nutritional requirements of as many different *Lactobacillus* types as possible (e.g. Cox & Briggs, 1954) considerably reduces the chance of such selective action by the medium.

Experiment 4. To examine more closely the occurrence of cross-contamination between the different lactobacilli used in experiments of the type described in Expt. 1, tests were made using a strain of *Lactobacillus casei* and one of *L. fermenti* whose colony forms upon TJ agar were clearly recognizable. After each subculture and washing, the control and washed cultures were plated upon TJ agar and checked after growth for 48 hr. at 37° for the presence of foreign colonies. Such colonies were isolated and classified.

Results. Contamination between the two strains was readily detected in the washed series, and each contaminated the other. No contamination occurred in the control unwashed series during ten subcultures.

Experiment 5. One strain of *Lactobacillus casei* and one of *L. fermenti*, each derived from single cell isolates were subcultured in four parallel series in TJ broth at 48 hr. intervals. One series was an unwashed control, one was triple washed and resuspended in 0·85% saline solution, one was similarly treated with 0·05% manganous chloride solution, and one with quarter strength Ringer's solution between each subculture. Manganous chloride was chosen as a possible agent of genetical mutation, and Ringer's solution as a relatively non-toxic suspending fluid. In all, 24 successive subcultures and washings were made for both strains in each series.

Result. From observations of colony forms as in Expt. 4 and by their subsequent classification, contamination was detected in all three series of washed cultures after less than ten transfers. The unwashed series was still pure at the 10th subculture, but contamination did finally occur at the 23rd subculture.

Although Coolidge *et al.* (1949) reported no changes in cultures washed in solutions other than 0·85 % sodium chloride, we consider that the actual fluid employed is of little significance in the initiation of 'changes of character'. The important factor is the exposure to contamination by excessive manipulation of the cultures examined.

DISCUSSION

The 'stabilizing effects' of prolonged artificial culture upon *Lactobacillus* strains reported by Howitt (1930) and by Harrison (1942) were almost certainly due merely to the gradual purification during maintenance, of strains which were initially impure. Harrison's (1942) observations of changes were similarly the result of the use of mixed cultures, a fact which Harrison realized was possible, but which was not investigated.

In Coolidge's work, the use of single cell isolates precludes the possibility of initial impurity (see Expt. 3, above), but the use of both homofermentative and heterofermentative strains in the experiments, and contamination during the subculturing and washing of cultures is considered by the present authors to be the true explanation of the apparent metabolic changes observed. That these workers did not detect the mixed nature of the supposedly changed cultures was probably because of the complete overgrowth of the contaminant strain and loss of the original culture type, or the lack of techniques suitable for the adequate diagnosis of strains, or both. It also appears certain that the process of centrifuging and washing cultures, although conducted with normal bacteriological precautions, facilitates contamination; the actual solution used for the washing having little or no affect upon the result. Another possible factor assisting contamination in the present study was the use of screw-capped bottles. In these, the carbon-dioxide generated by heterofermentative strains causes a distinct effervescence when shaken rapidly and opened and the bacteria may be disseminated by atomization.

In the taxonomy of *Lactobacillus*, the acceptance of morphological variability as a characteristic of the genus has undoubtedly led to the description of variation in cultures which were, in fact, simply impure. In consequence, such studies have produced apparent anomalies which only serve to confuse the systematics of the group. Laxminarayana *et al.* (1952) reported heterofermentative strains of *L. plantarum* which, in our opinion, were no more than cultures containing lactobacilli of both the *L. casei* and *L. fermenti* type. Mixtures of this type have been encountered in this laboratory, and their combined characters can be mistakenly interpreted as those of gas producing strains of *L. plantarum*. In our experience all such cultures have subsequently been resolved into two pure cultures by normal purification techniques.

So far as we know, no other investigations of the phenomena reported by Coolidge *et al.* (1949) have been attempted. The interpretation of our own results as due to contamination naturally leads us to believe that the original reports are similarly explicable. The possibility of a genetical change, especially one incurred by the action of saline solution, appears most doubtful, but cannot be entirely ignored. Little or nothing is known of the genetics of *Lactobacillus*, and because of the extreme importance of such variations, if in fact they occur, some introductory experiments were made to determine whether genetically 'marked' strains of heterofermentative lactobacilli could be persuaded, either by mutation or back-crossing to produce homofermentative variants. These were uniformly negative and will not be described here in detail.

Our findings agree with those of Rogosa *et al.* (1953) that major variations in taxonomic characters, such as a change from the heterofermentative to the homofermentative type which would imply the instability of the main subgeneric division of the genus (Davis, 1955), do not normally occur. We wish to emphasize that for the purposes of primary purification of lactobacilli, the morphological characters of colonies and cells may be reliably employed as indicators of strain uniformity, as well as inspecies diagnosis.

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Studies on *Cytophaga fermentans*, n.sp., a Facultatively Anaerobic Lower Myxobacterium

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SUMMARY: A previously undescribed myxobacterium was isolated from marine mud and studied in pure culture. This organism is a typical non-fruiting, amicrocystogenous lower myxobacterium and has been classified in the genus *Cytophaga* under the name *C. fermentans*, n.sp., to indicate its ability, unique among the cytophagas so far studied, to grow anaerobically at the expense of organic compounds. The organism can utilize as carbon and energy sources simple sugars and starch, but not cellulose, agar or chitin. Ammonium salts, asparagine or glutamine are satisfactory nitrogen sources; urea, nitrates and amino acids are not. Thiamine and relatively high concentrations of iron and CO_2 are required for growth in a defined medium. Glucose is fermented to acetic, propionic and succinic acids, in approximately equimolar quantities, plus a small amount of lactic acid. In addition, a large quantity of mucilage, which appears to be a nitrogen-containing substance, is produced during the fermentation.

The myxobacteria may be readily distinguished morphologically from the true bacteria and from almost all other micro-organisms. The flexible vegetative cells, lacking flagella or a rigid cell wall and showing gliding motility on solid surfaces, distinguish all members of the group.

Classification of the higher myxobacteria has been based mainly on the morphology of their fruiting structures. The lower myxobacteria, in contrast, are largely indistinguishable from one another morphologically, and all non-fruiting, amicrocystogenous forms are assembled in the one genus *Cytophaga* (Stanier, 1942). Biochemical characteristics of the higher myxobacteria have been very little studied, largely because of the difficulties of growing them in well-defined media, although recent work has indicated that these difficulties may not be as great as was formerly supposed (Oxford & Singh, 1946). The lower myxobacteria, on the other hand, have proved more amenable to experimental study, and it has become increasingly apparent that they comprise many types of great biochemical diversity. Future classification will, therefore, doubtless be based on physiological criteria.

The best known lower myxobacteria are the cellulose-decomposing cytophagas, an ecological-physiological group for which adequate enrichment techniques exist (Fåhraeus, 1947). Less well known are the chitin-decomposing soil forms (Stanier, 1947) and the agar-decomposing marine forms (Stanier, 1942). A number of lower myxobacteria has also been described which attack none of these complex substances and about whose ecology and physiology little is known (Imsenecki & Solnzeva, 1945; Starr, 1953).

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Information that there exists in marine muds a facultatively anaerobic agar-decomposing lower myxobacterium presented an interesting new aspect of the biochemical diversity of the group, as all previously studied myxobacteria have been considered to be aerobic. Both Dr S. R. Elsden and Dr H. Larsen (personal communications) have encountered such a myxobacterium, tentatively identified as a cytophaga, in enrichment cultures aimed at the isolation of green bacteria. The present work was begun in an effort to re-isolate and characterize this organism, which was lost before it could be studied. Although Elsden's and Larsen's organism was not found by the writer, an equally interesting facultatively anaerobic lower myxobacterium was obtained and has been studied in some detail. The characterization of this organism is the subject of the present report.

Isolation and cultivation

The organism was isolated from a bottle enrichment culture similar to that employed by Larsen (1952) for the enrichment of green sulphur bacteria and in which he found the anaerobic cytophaga previously mentioned. The medium had the following composition (g.): NaCl, 3; $NaHCO_3$, 0·2; KH_2PO_4, 0·1; NH_4Cl, 0·1; $MgCl_2$, 0·05; $FeCl_3 \equiv 50$ μg.Fe; tap water to 100 ml.; pH 7·0. Powdered agar was added to the bottles as a source of carbon and energy, since Larsen's and Elsden's organism had proved to be an agar-decomposer. Many bottle cultures were prepared using this and similar media and inocula from various marine sources. In a number of these cultures myxobacterium-like cells were seen, but only in one did a myxobacterium develop to the extent of becoming the predominant organism.

After a number of serial transfers through bottles of the above medium, the successful enrichment culture was streaked on plates of rich organic media containing peptone or yeast extract. On most of these plates a rapid development of spreading eubacterial forms occurred and no myxobacteria were seen. On one series of plates, however, composed of the original enrichment medium +0·5% peptone and 2% agar and lacking the $NaHCO_3$, a myxobacterium slowly developed as almost the only form. Successive streak plates demonstrated that the organism was in pure culture. The yellow colonies lay in shallow craters in the agar, in contrast to the organism seen by Larsen and Elsden, which was reported to produce a pink pigment and to decompose agar rapidly and extensively.

Subculture by streaking on plates of a wide variety of organic media soon demonstrated that development was most satisfactory in a medium containing 1% yeast extract. The following medium (medium A) was used for the maintenance of stock cultures throughout most of this work (g.): yeast extract, 1; NaCl, 2; K_2HPO_4, 0·02; $MgSO_4$, 0·05; NH_4Cl, 0·1; $FeCl_3 \equiv 50$ μg. Fe; tap water to 100 ml.; pH 7·4. For slope cultures and stab cultures, both of which were carried at all times, 2 and 1% agar were added, respectively. Cultures were transferred weekly and were incubated at 30° or at room temperature.

Although the method of isolation encouraged hopes that the organism could be obtained at will by use of an enrichment culture technique, efforts to repeat this isolation procedure or to develop a more reliable method for enrichment have failed. It appears that not enough is known about the organism at present to enable one to duplicate those conditions under which it thrives in nature and, conversely, the isolation gives no clue as to its biochemical role in nature.

Nutrition and physiology

This myxobacterium is at present unique in its group by virtue of its ability to develop anaerobically and it has accordingly been named *Cytophaga fermentans* n.sp.

The growth requirements of *Cytophaga fermentans* are relatively simple. As shown in Table 1, only a few of the simple carbohydrates and starch are suitable as carbon and energy sources. Neither cellulose, agar nor chitin will support growth. This organism, like the agar- and chitin-decomposing cytophagas described by Stanier (1942, 1947) does not show the sensitivity to autoclaved glucose exhibited by the cellulose-decomposing soil forms (Stanier, 1942; Fåhraeus & Sijpesteijn, 1949). Ammonium salts, glutamine and asparagine are suitable nitrogen sources. Nitrates, urea and amino acids are not utilized.

Table 1. *Ability of* Cytophaga fermentans *to utilize various compounds as carbon and energy sources as determined by growth in defined medium B without glucose, with addition of* 1 % *of the compound tested, under semi-anaerobic conditions*

Utilized	Not utilized	
Glucose	Galactose	Aspartic acid
Fructose	Rhamnose	Glutamic acid
Sucrose	Trehalose	Alanine
Lactose	Dulcitol	Lactic acid
Maltose	Sorbose	Pyruvic acid
Cellobiose	Ribose	Succinic acid
Xylose	Alginic acid	α-Ketoglutaric acid
Arabinose	Cellulose*	Fumaric acid
Mannose	Chitin	Malic acid
Mannitol	Agar	Acetic acid
Raffinose	Glycerol	Citric acid
Starch	Ethanol	

* Cellulose added to the medium in the form of a filter-paper strip. The medium in all cases was adjusted to pH 7·2–7·4.

The only organic growth factor required is thiamine and the only specific mineral requirement detected is for iron in relatively high concentration. A fairly high concentration of CO_2 (supplied here as *c.* 1 % $NaHCO_3$) is required for continued development of the organism in a defined medium. This requirement could be replaced by yeast extract but not by any of the organic compounds most commonly found to substitute for CO_2 requirements in other organisms, e.g. succinate, malate, fumarate, citrate, glutamate. As was found for *Escherichia coli* by Lwoff & Monod (1949), CO_2 must here be essential for the formation of still other metabolites in the cell.

NaCl in concentrations from 2 to 3% permits good growth immediately, but the organism can be 'trained' to grow in concentrations as low as 1%. NaCl in the defined medium can be replaced by KCl with no deleterious effects. On the whole, a fairly wide variation in the balance of minerals in the medium can be tolerated. The temperature range for growth is quite broad, the optimum being around 30°. The optimum pH range for the initiation of growth in the liquid defined medium is from 6·7–8·3, again, a fairly wide range being tolerated.

The defined medium (medium B) used throughout the metabolic studies on *Cytophaga fermentans* has the following composition: glucose, 1 g.; NaCl, 2 g.; NH_4Cl, 0·1 g.; KH_2PO_4, 0·02 g.; $MgSO_4$, 0·05 g.; $FeCl_3 \equiv 50$ μg. Fe; thiamine HCl, 500 μg.; Pyrex glass-distilled water, 78 ml.; HCl (conc.), 1 ml.; bromthymol blue, 5 ml. of a 0·1% solution. The medium is tubed in 6·4 ml. amounts and sterilized; the glucose is thus autoclaved in an acid solution and therefore has less tendency to caramelize. Just before inoculation, 1·6 ml. of a sterile 6·25% $Na_2CO_3.H_2O$ solution is added aseptically to each tube, bringing the pH value to *c.* 7·4. After inoculation the cotton stopper is pushed down into the tube and a rubber stopper inserted to prevent the escape of CO_2. This medium has proved satisfactory through over 500 consecutive transfers. Maximum growth (*c.* 2×10^9 cells/ml.) is attained in about 48 hr. at 30° and is accompanied by a sharp drop in pH value because of acid production. The cells do not remain viable long after pH 6·0 is reached; cultures in this medium are therefore transferred at about 48 hr. intervals and relatively large inocula are used.

A surprising result of the nutritional studies on *Cytophaga fermentans* is the unsuitability of agar as a carbon and energy source. The production of gelase fields and craters on the surface of agar plates and the softening of the underlying substrate would suggest agar utilization. Moreover, a solid medium which contains yeast extract + minerals + agar supports growth, while a liquid medium containing yeast extract and minerals alone will not. Yet the addition of washed agar to the defined medium B (minus glucose) did not permit proliferation. Experiments with agar extracts indicate that impurities in commercial agar are sufficient to support a fair growth when the agar is present in 2% concentration. It must be concluded that the organism is not, as was first assumed, an agar decomposer in the sense that it can grow at the expense of this compound. The fact that galactose, the unit saccharide of agar, also was not suitable as a carbon and energy source lends support to this conclusion.

Morphology

Cytophaga fermentans grows on the surface of solid media in a manner sufficiently characteristic to differentiate it from almost all eubacterial forms. The first growth to appear upon transfer to a solid medium is a thin film, only a few cells thick at most and visible only by reflected light, which spreads out over the surface from the points of inoculation. When pieces of inoculum are placed gently upon the surface of the agar, the film spreads in all directions at a fairly uniform rate. When, however, inoculation is performed by streaking, the growth spreads more rapidly along the streaks made by the inoculating instrument than it does across the medium in a direction perpendicular to the streaks. Stanier (1942) studied the tendency of myxobacteria to migrate along lines of stress which have been induced in an agar substrate and coined the name 'elasticotaxis' for this oriented movement, the mechanism of which is still not understood.

After 4 or 5 days of incubation the character of the surface growth has changed conspicuously in the central portion of the 'colonies'. Many cells are collected into large swirls which appear at random throughout the film and which gradually increase in thickness until, after about 2 more days, the surface of the growth is dotted with large, shiny, bright yellow mucilaginous blobs of irregular shape. These blobs, which are translucent or opaque, sometimes give the appearance of isolated eubacterial colonies because of the relative transparency of the film on which they lie (Pl. 1, fig. 1). During the development of this surface growth a change in the underlying agar has occurred so that the colony lies in a broad, shallow crater which extends *c.* 2–3 mm. beyond the advancing colony edge. Co-extensive with the crater is a zone of clearing in the agar, otherwise slightly cloudy.

A microscopic examination of the organisms of which these colonies are composed yields the following picture. Cells from the advancing colony edge are slender flexible rods of low refractility with rounded ends, *c.* 5–15 μ. long and *c.* 0·6 μ. wide. The length of the cells is fairly uniform in a given area (Pl. 1, fig. 2). In material taken from the blobs in the central portion of a colony the cells are longer and more variable in length. They are there usually *c.* 15–30 μ. long, although in very old cultures 'cells' up to 50 μ. in length have been observed. The cell width appears to remain constant under all conditions.

In the older portions of the colonies there are usually many involution forms. The S, U, O and V forms described in other cytophagas by previous workers occur frequently. Branching cells have never been seen in *Cytophaga fermentans*. The involution forms most frequently observed are small round bodies of low refractility, whose diameter extends from that of the vegetative cells (*c.* 0·6μ.) up to 2·5μ. It seemed likely at first that these round bodies represented later stages of a life cycle of the organism; the fact that they appear in great numbers in almost all old cultures argues for this interpretation. However, their lack of refractility and their variability in size do not correspond with the appearance of the microcysts formed by other myxobacteria. In addition, old cultures containing many round bodies are very apt to be non-viable when transferred, which would support the view that these are non-viable cells which have lost the ability to maintain their rod shape. Under none of the conditions under which it was studied could *C. fermentans* be induced to form typical microcysts, which led to the conclusion that it is truly a 'lower' myxobacterium.

Cells grown in the synthetic medium do not produce the yellow pigment present in the surface colonies on agar media. The cells are shorter than those occurring on solid medium and quite uniform in a given culture (8–10μ.); in older cultures cell length increases to 15–20μ. The involution forms most frequently observed are the round bodies, which increase in number with the age of the culture and which may eventually outnumber the healthy vegetative cells.

A curious colonial variant was produced when liquid cultures in medium B were streaked on plates of medium A. The resulting growth consisted of small, bright yellow, compact, opaque, convex, round, glistening colonies with an even edge. After growth for a week these colonies were only 1–2 mm. in diameter, and were sunk in craters *c.* 2–4 mm. in diameter. Such colonies resemble those of many eubacterial species, their only distinctive property being the craters produced in the agar substrate (Pl. 1, figs. 3, 4). The cells from such colonies, however, are indistinguishable from those from the normal colony type. The variant type is unstable and usually reverts, gradually, to the spreading type of growth. Stanier (1942, 1947) observed a similar variant colony type in a number of cytophagas studied by him.

Cytophaga fermentans exhibits on solid surfaces typical myxobacterial gliding motility accompanied by continuous flexing movements of low amplitude. No instance has been observed of cells moving in co-ordinated units such as Stanier (1942) found in the swarm extensions of *Sporocytophaga myxococcoides*. In liquid media the cells are non-motile but show vigorous flexing movements, particularly those cells one end of which has become stuck to the surface of a slide or coverslip.

The pigment produced by *Cytophaga fermentans* under aerobic conditions turns blue in the presence of concentrated sulphuric acid, can be extracted from the cells by methanol and then extracted from 90 % methanol by light petroleum. The absorption spectrum is that of a carotenoid, resembling that of β-carotene.

Fermentation of glucose

Materials and methods. The fermentation of glucose was studied in bottle cultures of *Cytophaga fermentans* growing in defined medium B. The acid produced during the fermentation liberated such large quantities of CO_2 from this bicarbonate-rich medium that it did not seem feasible to determine accurately the quantity of CO_2 produced or taken up by the organism during dissimilation of the substrate. Therefore only the non-gaseous end-products of the bottle fermentations were analyzed.

Medium B (1500 ml.) minus the indicator was prepared and sterilized; a solution of Na_2CO_3 was autoclaved separately. Just before inoculation, the pH value of the flask of medium B was adjusted to pH 7·4 by the gradual addition of sterile Na_2CO_3. An inoculum consisting of *c.* 25 ml. of a 48 hr. culture in the same medium was then quickly added, and the flask was swirled to give thorough mixing. Enough of the contents was then poured into a sterile litre Pyrex bottle to fill it to the brim so that, upon insertion of the ground glass stopper, fluid was forced out of the neck of the bottle and no air space was left inside. A determination of the initial glucose was immediately performed on the excess inoculated medium, using the Luff-Schoorl method (Browne & Zerban, 1941) modified for use within the range of 1–10 mg. glucose.

The bottles were incubated in a constant temperature room at 29°. Evolution of gas became apparent within 24 hr., and by the end of 48 hr. the cultures usually reached maximum turbidity. When the bottles were removed from the incubator, glucose determinations were immediately performed. Simultaneously, portions of the fermentation liquor were centrifuged for 1 hr. in a Servall high-speed angle centrifuge kept in a cold room at 14°. The 'sediment' obtained by this centrifugation consisted of a slimy mass of viscous material of approximately one-third the total volume of the fluid centrifuged. The supernatant was again centrifuged, a very slight amount of sediment being obtained this time. The two sediments were combined and treated with 1·6 vol. of 95% ethanol (v/v) in water. The copious white stringy precipitate formed upon addition of ethanol was washed twice in 95% ethanol, once in absolute ethanol and once in ether. It was then vacuum dried in a weighed centrifuge tube to constant weight. A sample of the final supernatant fluid was treated with 1·6 vol. of 95% ethanol and the very slight amount of precipitate formed was treated in the same manner as the precipitate discussed above. These two precipitates were considered to represent the total amount of slime and cellular material produced during the fermentation. No method for separating the cells from the slime has as yet been devised.

A sample of the supernatant fluid was steam-distilled after acidification and the total amount of steam-volatile acids was determined by titration. The nature and ratios of the volatile acids present were determined by performing a Duclaux distillation on the steam-distillate (Knetemann, 1928). The residue from the steam-distillation was extracted with ether in a continuous liquid-liquid extractor and the ether extract was analyzed for lactic acid by the method of Friedemann & Graeser (1933) as modified by Stone (1938). Succinic acid was determined manometrically on the untreated fermentation fluid by using a beef heart succinic dehydrogenase preparation according to Cohen (Umbreit, Burris & Stauffer, 1949).

The 2, 3-butyleneglycol occasionally found among the fermentation products was detected by the method of Kniphorst & Kruisheer (1937). The presence of trace amounts of keto acids was detected by the method of Friedemann & Haugen (1943). Paper chromatography of the 2, 4-dinitrophenylhydrazone derivatives by the method of Cavallini, Frontali & Toschi (1949) revealed the presence of three compounds: the derivatives of pyruvic and α-ketoglutaric acids and that of an unknown keto compound which moved just ahead of the pyruvic phenylhydrazone on the chromatograms. Since the 2, 3-butyleneglycol and keto acids were present only in trace amounts, they were not determined quantitatively.

The presence of glycerol was not demonstrated in the fermentation fluid using the periodate method of Neish (1950). In cultures in which essentially all of the glucose had been decomposed, the supernatant liquid from the centrifugations was made alkaline and distilled. The distillate was tested for the presence of alcohols (and aldehydes) by the dichromate method given by Neish (1950). No alcohol or aldehyde was detected in these cultures.

Results. A number of fermentation liquors of different ages with different initial substrate concentrations were analyzed. The results of three representative fermentation analyses are given in Table 2. It can be seen that *Cytophaga fermentans* performs a fermentation essentially of the propionic acid type. The chief end-products are the ethanol-precipitatable material, consisting of slime + cells, and acetic, propionic and succinic acids. The ratios of the three major acidic end-products varied considerably from one fermentation to another, although the fermentations were carried out under conditions as nearly identical as possible. A small quantity of lactic acid was produced; as a rule the quantity did not vary much from fermentation to fermentation.

Table 2. *Fermentation balances on bottle cultures of* Cytophaga fermentans *growing in defined medium B*

Experiment	1	2	3
Glucose added to medium (%, w/v)	1	0·5	1
Available glucose disappeared (%)	92	100	84

	Amounts of products/100 μmole glucose utilized					
Products	μmole	μAC*	μmole	μAC	μmole	μAC
Slime + cells (as glucose)	16	96	22	132	15	90
Acetic acid	48	96	45	90	36	72
Propionic acid	48	144	55	165	44	132
Succinic acid	48	192	39	156	55	220
Lactic acid	5	15	5	15	15	45
Keto acids	Trace	.	Trace	.	Trace	.
Acetoin	Trace	.	—	.	—	.
Total μA carbon	543		558		559	
Carbon accounted for (%)	91		93		93	
Redox balance†	1·00		1·03		0·98	

* The symbol 'μAC' is used to express microgram atomic weight. It bears the same relation to gram atomic weight as does μmole to gram molecular weight.

† Calculated according to the method of Barker & Lipmann (1944).

The analysis of a bottle fermentation in which the Na_2CO_3 and HCl of the medium were replaced by M/15 phosphate buffer (pH 7·0) gave the following quantities of acids/100 μmole of substrate utilized: acetic 45 μmole, propionic 60 μmole, succinic 22 μmole. Thus, in a medium essentially lacking in CO_2, less succinic acid was formed, proportionally; a finding which suggests the involvement of CO_2 fixation in the production of this compound.

The dry weight of slime and cells produced accounted for as much as 25% and in no case less than 15% by weight of the glucose dissimilated. The chemical composition of slime + cells was considered to be the same as that of the substrate (CH_2O) for the purpose of calculating fermentation balances. An analysis of the ethanol precipitate of slime + cells gave the following figures:

carbon 45%; hydrogen 6·95%; nitrogen (Dumas method) 9·45%; oxygen, by difference, 38·6%. This corresponds closely to the formula $C_6H_{11}O_4N$. The nitrogen content is close to that of whole bacterial cells (Buchanan & Fulmer, 1928) which implies that the slime itself must be a nitrogen-containing substance. Protein determinations based on the biuret reaction and performed according to the method of Weichselbaum (1946) modified by Dr A. P. Pardee (personal communication) indicated that the mixture of slime + cells contained 70–90% protein. A general test for carbohydrates based on the Molisch reaction (Kabat & Mayer, 1948) showed *c.* 10–15% carbohydrate in the mixture (calculated as glucose). Although the formula $C_6H_{11}O_4N$ cannot represent more than an approximation of the average composition of the slime, it serves to show that the practice of considering it as 'carbohydrate' for the calculation of redox balances cannot have resulted in appreciable discrepancies.

Taxonomy and diagnosis

Cytophaga fermentans has been assigned to the order Myxobacteriales by virtue of its extremely flexible slender cells of low refractility, lacking a rigid cell wall and showing flexing and gliding motility. The only other groups which possess these morphological characteristics are the gliding Vitreoscillaceae described by Pringsheim (1951), and the blue green algae. These three groups, the Myxobacteriales, the Myxophyceae and the Vitreoscillaceae, seem therefore closely related, but much more will have to be known about the members of all three groups before the nature of their relationships can be clearly formulated and before one can with assurance classify all organisms showing the above characteristics.

The myxobacteria which do not possess a complex life cycle are classified in the family Cytophagaceae (Stanier, 1942). The members of this family are all included in one genus, *Cytophaga*, and so far no obvious natural separations indicating the need for more than one genus have been detected. Although it seems likely that as more non-fruiting myxobacteria are isolated and studied it will be convenient to place them in groups made along the lines of ecological types, such as the cellulose-decomposing soil forms and the marine agar-decomposing forms, for example, it seems wise for the time being to avoid confusion by keeping the classification as simple as possible and by refraining from making further taxonomic divisions before the significance of the criteria used can be fully assessed.

For these reasons the organism has been classified as a member of the genus *Cytophaga*. To emphasize its distinctive ability to develop anaerobically at the expense of sugars, it has been named *Cytophaga fermentans* n.sp. The description is as follows.

Morphology: slender flexible rods of low refractility with rounded ends. Width 0·5–0·7μ.; length extremely variable, averaging 8–15μ. in young culture to 30μ. in old cultures. Extremely long forms occasionally seen. Gram-negative. Stains evenly with crystal violet or carbol fuchsin. Young cells show gliding motility on surfaces and active flexing.

Growth on yeast extract agar plates: a thin smooth glistening transparent film, spreading over agar surface. Later, mucilaginous opaque bright yellow blobs of various sizes and shapes appear at random in central portion of film. Colonies lie in shallow craters extending *c.* 1–2 mm. beyond advancing colony edge. Cells on agar surfaces display elasticotaxis. An unstable variant colony type appearing after passage through defined liquid medium consists of round, glistening, convex, bright yellow, mucilaginous colonies with entire edge.

Growth in yeast extract agar stabs: a diffuse cylinder of creamy white growth spreading out from line of inoculation.

Carbon and energy sources: under anaerobic conditions simple sugars (including glucose, sucrose, lactose, maltose) and starch utilized. Cellulose, agar, and chitin not utilized.

Nitrogen sources: ammonium salts, asparagine and glutamine utilized. Nitrates, amino acids and urea not utilized.

Growth factors: thiamine required. High CO_2 tension required for continued growth in defined medium.

Nitrites not produced from nitrates.

Catalase formed.

Optimum salt concentration range: 2·5–3% NaCl.

Optimum temperature: Around 30°.

Optimum pH value: Around 7–8.

Facultatively anaerobic. Glucose fermented with production of acetic, propionic and succinic acids in approximately equimolar quantities. Large quantity of mucilage and small amount of lactic acid also produced. The pigment characteristic for cells grown aerobically on yeast agar is not produced under anaerobic conditions.

Source: Marine mud.

Habitat: Probably decaying marine vegetation.

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Amino Acid Metabolism in the Genus *Bordetella*

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SUMMARY: *Bordetella pertussis*, *B. parapertussis* and *B. bronchiseptica* all grow in an amino acid medium, using glutamate and forming ammonia from it. After the glutamate has been used, *B. pertussis* and *B. parapertussis* deaminate aspartic acid, serine, glycine, alanine and proline. *B. bronchiseptica*, as well as these amino acids, deaminates threonine, valine, methionine, the leucines and phenylalanine, leaving only histidine, lysine and arginine. *B. bronchiseptica* grows the most rapidly of the three and *B. pertussis* the most slowly. All three organisms oxidize glutamate to give CO_2 and ammonia but in each some nitrogen is unaccounted for. *B. bronchiseptica* contains a glutamic decarboxylase and *B. parapertussis* forms a pink pigment from tyrosine.

The genus *Haemophilus* originally contained organisms which required blood for growth. As the true requirements of the species were found, it became clear that blood was required by different members for different reasons. The influenzae-parainfluenzae-canis group needs haemin and coenzyme I, but *H. pertussis* grows in the absence of both these compounds (Fildes, 1923). The precise function of blood in the growth of *H. pertussis* is not yet known. *H. parapertussis* and *H. bronchisepticus*, although they do not need blood for growth, resemble *H. pertussis* and differ from the *H. influenzae* group in not using carbohydrates (see *Topley & Wilson's Principles*, 1946; Jebb & Tomlinson, 1951) and the three species are also antigenically related (Ferry & Noble, 1918; Bradford & Slavin, 1937). López (1952) has suggested that *H. pertussis*, *H. parapertussis* and *H. bronchisepticus* should be put together in a new genus, *Bordetella*. Proom (1955) showed that the growth requirements of the three species are similar, but the metabolism has not yet been adequately investigated. It was to compare the amino acid metabolism of these species that the present work was undertaken.

METHODS

Organisms. Virulent strains of *Bordetella pertussis* were obtained from several public health laboratories and dried by the method of Greaves (1944) at the third subculture after isolation. They were maintained on Bordet Gengou medium (BG) and discarded at the 14th subculture, when another dried culture was opened. The strains of *B. parapertussis* C 803, C 691, NCTC 8250 NCTC 7385 and *B. bronchiseptica* NCTC 8752, 8760, 8762 were maintained on digest agar (DA). All cultures were incubated at 35°.

Growth experiments. Growth experiments were carried out in a liquid medium similar to that of Cohen & Wheeler (1946) except that cysteine and yeast extract were sterilized by filtration and added to the medium after it had been autoclaved (C.W. 1). Yeast extract was made by extracting dried yeast (Standard Yeast Co.) for 15 min. with an equal weight of water at 100° and used at a concentration of 1 in 400. The amino acid source was Difco vitamin-free Casamino acids containing 36 % amino acid (dry weight). The pH value of C.W. 1 medium after autoclaving was 6·9–7·0. Inocula of *Bordetella pertussis* were grown for 24 or 48 hr. on BG plates and inocula of the other organisms on DA medium. The cells were suspended in phosphate saline (pH 7) and 0·02 ml. added to each 20 ml. C.W. 1 medium to give a final concentration of about 10^{-7} mg. dry weight/ml., or 10^3 viable cells/ml. The medium was contained in 100 ml. Erlenmeyer flasks capped with 30 ml. beakers and incubated on a horizontal shaker (Kantorowicz, 1951). Flasks were removed at intervals and stored at 4°. With cultures of *B. bronchiseptica*, samples were removed for turbidity estimation and the rest of the culture heated at 65° for 15 min. to inactivate glutamic decarboxylase.

Washed suspensions. Organisms were grown in 250 ml. quantities of C.W. 1 medium contained in penicillin flasks. *Bordetella pertussis* was grown for 48 hr. and *B. bronchiseptica* for 24 hr. from an inoculum of about 0·1 mg. dry wt. organisms/250 ml. With this size of inoculum, growth of *B. parapertussis* was poor after 24 hr. and, after 40 hr. incubation, oxygen uptake was slow; cultures grown for 24 hr. from an inoculum of 1 mg. dry wt./250 ml. were therefore used. The organisms were spun from the medium, washed in phosphate saline (pH 7) and resuspended in an appropriate buffer.

Oxidation of glutamate. Washed suspensions of organisms were incubated at 37° in Warburg manometer flasks of about 20 ml. capacity having one or two side arms. The suspension (3–7 mg. dry wt.) was put in the side arm and glutamate (final concentration 0·0087 M) in the main compartment, each being made up in 0·11 M-phosphate buffer to give a final volume of 2·3 ml. CO_2 output and O_2 uptake were measured by the method of Warburg & Yabusoe (1924). Oxygen uptake was estimated in four cups and, when the reaction was virtually over, the contents of these were pooled, heated at 65° for 15 min. and stored at 4° until ammonia and glutamate estimations and amino acid chromatograms were carried out. Rates of reaction were expressed as Q_{O_2} (μl. O_2 used/mg. dry wt. organisms/hr.).

Decarboxylation of glutamate. Washed suspensions (2–6 mg. dry wt. in 3·5 ml.) were incubated in manometer flasks with 0·2 M-acetate buffer containing 0·0057 M-glutamate and 0·09 % (w/v) cetavlon. CO_2 output was measured directly in air at 37°.

Viable counts were made by the method of Miles & Misra (1938) using BG plates for all organisms.

Concentration of suspensions of organisms was measured by a Gambrell photoelectric colorimeter with a minus red filter. The dry weight was related to turbidity by means of an empirical calibration curve. A suspension containing 0·20 mg. dry wt./ml. had the same turbidity as the American N.I.H. *Haemophilus pertussis* vaccine standard of $10{,}000 \times 10^6$ organisms/ml. Nitrogen estimation by micro-Kjeldahl gave 12 % nitrogen for each species.

Ammonia was estimated with boric acid in Conway units according to Hawk, Oser & Summerson (1949).

Glutamic acid estimation. Glutamate was estimated with the decarboxylase of *Clostridium welchii* S.R. 12 using 0·05 % octanol in 0·2 M-acetate buffer pH 4·9 (see Krebs, 1948).

Paper chromatographs. Culture (0·01 ml.) was applied to Whatman no. 1 paper, and one-way chromatograms were run overnight using *n*-butanol + acetic acid + water in the proportion of 5 : 12 : 3 and sprayed with 0·1 % ninhydrin in 90 % butanol containing 0·1 % collidine (see Woiwod, 1949).

RESULTS

Metabolism during growth

Rate of growth in liquid medium. The C.W. 1 medium was used for these experiments because *Bordetella pertussis* requires an inoculum equivalent to *c.* 10^{-4} mg. dry wt. organisms/ml. for consistent growth in the original Cohen & Wheeler medium containing autoclaved cysteine, and, with such a large inoculum, it is difficult to obtain satisfactory replicates. When the cysteine is sterilized by filtration, organisms equivalent to 10^{-7} mg. dry wt./ml. are an adequate inoculum. Growth experiments were carried out with three strains each of *B. pertussis*, *parapertussis* and *bronchiseptica*. The results obtained within a species were consistent; a representative experiment is recorded in Fig. 1 and Tables 1 and 2. *B. bronchiseptica* grew most quickly, having the shortest lag phase and mean generation time (M.G.T.) in the log phase (Table 1). It grew at a constant rate until the maximum turbidity was almost reached and then the rate decreased progressively (Fig. 1). *B. parapertussis* grew at a constant rate for about 20 hr. and then at half this rate (Table 1) for about 4½ generations (a 20-fold increase in dry weight) before growth finally stopped. *B. pertussis*, which grew the most slowly, also had a second slower logarithmic growth rate a quarter of the primary M.G.T. but it lasted for only 2½ generations or a six-fold increase in dry weight. The increase in turbidity with the last two organisms followed the same course as the viable count during the secondary growth phase but, in *B. bronchiseptica*, the turbidity increased sevenfold with a falling M.G.T. after the end of the logarithmic phase as determined by viable count. During the same period (24-80 hr.) the viable count only increased 2½ times (Fig. 1 and Table 2).

Table 1. *Rate of growth of* Bordetella pertussis, B. parapertussis *and* B. bronchiseptica

Cultures grown in 20 ml. C.W. 1 medium at 35°.

Organism and strain no.	Lag phase (hr.)	Primary M.G.T. (hr.)	Secondary M.G.T. (hr.)
B. pertussis M 2400	9	2·5	10
B. parapertussis C 691	9	1·7	3·3
B. bronchiseptica 8732	4	1	*c.* 20

Table 2. *Glutamate and amino acid metabolism during growth of* Bordetella *spp.*

Cultures incubated at 35° on shaker in Cohen & Wheeler medium containing 110–120 μl. glutamate/ml.

Organism	Time (hr.)	Turbidity (mg./ml.)	Glutamate used (μl./ml.)	Ammonia formed (μl./ml.)	Chromatogram showed
B. pertussis M 2400	55	0·015	1	2	.
Subculture 9	64	0·090	7	13	.
	72	0·23	53	51	Glutamate lighter
	79	0·38	111	110	Glutamate gone
	88	0·55	110	214	Glycine, serine, aspartic acid, alanine, proline lighter
B. parapertussis C 691	40	0·02	8	9	.
	44	0·05	21	22	.
	48	0·12	54	38	Glutamate lighter
	55	0·50	112	89	Glutamate gone
	64	0·90	112	203	Glycine, serine, aspartic acid, alanine lighter, proline gone
	72	0·95	—	262	Glycine, serine, aspartic acid, alanine lighter, proline gone. New spot above valine
B. bronchiseptica 8752	20	0·015	10	9	.
	24	0·11	15	15	.
	27½	0·35	50	49	.
	31	0·80	74	67	.
	35	0·95	112	111	Glutamate gone
	44	1·20	117	205	Glutamate gone, leucine less
	51½	1·30	—	274	All but basics and valine lighter

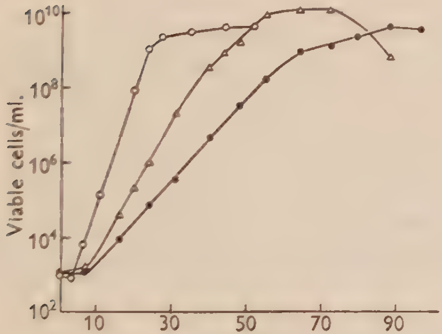

Fig. 1

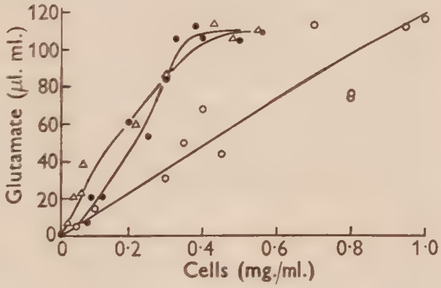

Fig. 2

Fig. 1. Rate of growth of *Bordetella bronchiseptica*, *B. parapertussis* and *B. pertussis*. Cultures grown in 20 ml. C.W. 1 medium at 35°. Results from same experiments as shown in Tables 1 and 2. *B. pertussis*, ●——●; *B. parapertussis*, △——△; *B. bronchiseptica*, ○——○.

Fig. 2. Relation of growth of *Bordetella* spp. to glutamate utilization. Cultures grown in 20 ml. C.W. 1 medium at 35°. Results from three strains of each species. *B. pertussis*, ●——●; *B. parapertussis*, △——△; *B. bronchiseptica*, ○——○.

Metabolism of glutamate during growth. In Cohen & Wheeler medium each species used glutamate for growth before other amino acids (see Jebb & Tomlinson, 1951). The growth rate of all three species fell when only about 10% of the total glutamate had been used and the pH value of the medium had risen from pH 7·0 to about 7·3. Growth of *Bordetella parapertussis*, but not of the other two organisms, continued at a steady rate for a short time after the rate of glutamate utilization had slowed down. The weight of cell substance formed by *B. bronchiseptica* for a given quantity of glutamate was greater than that formed by the other two organisms (Fig. 2). The ratio of ammonia formed to amount of organisms with *B. parapertussis*, however, resembled that with *B. bronchiseptica*. *B. pertussis* and *B. bronchiseptica* usually produced quantities of ammonia equivalent to the glutamate used (Table 2), but in a few experiments *B. bronchiseptica* formed slightly more ammonia than was accounted for by the glutamate disappearing. *B. parapertussis* formed slightly less than the expected quantity of ammonia.

Metabolism of other amino acids. After the glutamate had been used, ammonia formation continued at a decreased rate at the expense of other amino acids. *Bordetella pertussis* and *B. parapertussis* formed *c.* 300 μl. ammonia/ml. and aspartic acid, serine, glycine, glutamate, alanine and proline disappeared from the chromatogram (Table 2). Other amino acids were not used to an extent detectable on paper chromatograms. *B. bronchiseptica* deaminated the same amino acids and leucine, and finally only the basic amino acids were left, up to 400 μl. ammonia being formed. The only new nitrogen-containing compound showing up on the chromatograms was a spot above valine and methionine in the later stages of growth of *B. parapertussis*. It may have something to do with the brown pigment formed from tyrosine by this organism (see below) which only appears when growth is almost over.

It should be noted that in the key of amino acid positions on a chromatogram run in butanol + acetic acid given by Proom & Woiwod (1949), the amino acid in the crescent-shaped spot between the basic amino acids and glycine and serine (position C) is stated to be aspartic acid. However, when aspartic acid is run in a culture fluid of the kind used here it occupies the same position as glycine and serine; the amino acid which gives the crescent-shaped spot is arginine. When arginine is run in a salt solution such as phosphate buffer or a culture fluid it is concentrated into a very narrow line and has a slightly lower R_F.

Metabolism in washed suspensions

Oxidation of glutamic acid by washed suspensions. Washed suspensions of the three species oxidized glutamate with formation of ammonia (see Jebb & Tomlinson, 1951). The highest Q_{O_2} found for *Bordetella pertussis* and *B. parapertussis* was 100; *B. bronchiseptica* had a Q_{O_2} of up to 130. With each species the Q_{O_2} fell with increasing age of organisms. This was most noticeable with *B. parapertussis* which had a Q_{O_2} of, for example, 84 after 29 hr. growth and of 16 after 48 hr. growth. The relative quantities of ammonia and CO_2 formed and of oxygen and glutamate used are shown in Table 3. With each species, the ammonia formed was less than the quantity of glutamate used. Nitrogen-containing compounds were not apparent on chromatograms of suspensions of *B. pertussis* and *B. bronchiseptica*, but with *B. parapertussis* two compounds were present in the positions occupied by ornithine and arginine. These compounds were also found after metabolism of glutamate by two atypical strains of phase IV *B. pertussis*, G 154 E and G 146 E (Baddiley, Rowatt & Standfast, 1952). The ornithine-like compound was purified on columns of Amberlite IR 120 and IRA 400 and characterized as the dibenzoyl derivative. The melting point was the same as that of authentic ornithuric acid. The second spot had the same R_F as arginine when run in butanol + acetic acid and in phenol + NH_3, gave ornithine when heated at alkaline pH and was decarboxylated by the specific arginine decarboxylase of *Escherichia coli* 7020 (Gale, 1946) and consequently almost certainly was arginine. The quantity of these compounds was not estimated; it is not possible to say whether they account for all the missing nitrogen.

Table 3. *Glutamate metabolism by washed suspensions of* Bordetella *spp.*

Suspensions incubated in 0·11 M-phosphate buffer (pH 7) with 0·0087 M-glutamate at 37° in air.

Organism and strain no.	mole NH_3/ mole glutamate	atoms O/ mole glutamate	mole CO_2/ mole glutamate	atoms O/ mole CO_2
B. pertussis W 2043	0·76	5·3	3·2	1·60
B. parapertussis C 803	0·54	4·7	2·9	1·65
B. bronchiseptica 8760	0·62	4·8	3·1	1·5

Glutamic decarboxylase. Washed suspensions of *Bordetella bronchiseptica* when incubated with glutamate at pH 4·9 formed CO_2 and an amino compound which had the same R_F value as authentic γ-aminobutyric acid in butanol + acetic acid + water (5 : 1·2 : 5 and 8 : 1 : 10) and in butanol + water. The R_F of β-alanine is the same as that of γ-aminobutyric acid in the last two mixtures, but it is less in the first solvent (Synge, 1951). As with *Clostridium welchii* (Krebs, 1948) the rate of the reaction was increased by adding cetavlon, reaching a Q_{CO_2} of 250 with some strains. Glutamic decarboxylase was not found in any strain of *B. pertussis* or *B. parapertussis*.

Urease. Strains of the three species were tested for urease activity by the method of Clarke & Cowan (1952). *B. bronchiseptica* and *B. parapertussis* both contained the enzyme but none of the strains of *B. pertussis* did. This confirms the work of Ulrich & Needham (1953) on *B. bronchiseptica* and of López (1952) on all three species.

Formation of pigment from tyrosine. Bradford & Slavin (1937) showed that *Bordetella parapertussis*, unlike *B. pertussis*, formed a brown pigment during growth. Ensminger (1953) found that tyrosine was necessary for the formation of this pigment. Washed suspensions of *B. parapertussis* incubated with tyrosine formed a pink pigment which turned brown on prolonged incubation.

Glutamate increased pigment formation and the reaction was more rapid at pH 7·4 than at the lower pH values (Table 4). Three strains of *B. pertussis* and one of *B. bronchiseptica* incubated in this way did not form any pigment, nor was any formed during growth of either species.

Table 4. *Formation of pigment from tyrosine by washed suspensions of* Bordetella parapertussis

B. parapertussis 7385 equivalent to 0·95 mg. dry wt./ml. in 10 ml. 0·08 M-phosphate buffer at various pH values. Incubated 34 hr. at 37°. Colour measured as percentage absorption on Gambrell photoelectric colorimeter, with blue-green filter.

	pH value of suspensions		
	6·4	7·0	7·4
	Colorimeter reading		
Substrate			
None	13·5	15·5	16
0·01 M-glutamate	15	18	21·5
0·0011 M-tyrosine	14·5	16	22
0·01 M-glutamate 0·0011 M-tyrosine	17	31·5	60·5

DISCUSSION

It has been shown that *Bordetella pertussis*, *B. parapertussis* and *B. bronchiseptica* use glutamate for growth in preference to the other amino acids in casein hydrolysate. Some growth takes place on these amino acids but, since the growth rate falls when only a tenth of the glutamate has been used, growth at this stage must be limited by factors other than the glutamate concentration. We have not tested logarithmic growth on a medium deficient in glutamate, but Jebb & Tomlinson (1951) obtained poor growth in a medium without added glutamate even with so large an inoculum as 10^7 organisms/ml. Proom (1955) reported growth of *B. pertussis* in a medium without added glutamate, but gave no details of size of inocula or of amount of growth.

With each species tested here the growth rate fell when about 10% of glutamate had been used. The shape of the growth curves suggests that the medium is adequate for *Bordetella bronchiseptica*; that after some growth of *B. parapertussis*, a factor (or factors) is used up and growth is able to continue at a slower rate; and after some factor, possibly the same one, has been exhausted by *B. pertussis*, growth is poor and stops before a dry weight of organisms as large as with the other two organisms is reached.

The only amino acids not used by *B. bronchiseptica* are the basic amino acids. The high pH value obtaining at the end of growth (about 8·4) may prevent the metabolism of these amino acids. It is noteworthy that this organism contains a glutamic decarboxylase which acts at an acid pH value. γ-Amino butyric acid, the product of decarboxylation, is not present after oxidation of glutamate, nor, in any quantity, after growth.

The organisms use more glutamate for growth than any other compound and it is generally assumed that this substance is the energy source. In the medium used in these studies it cannot be the source of nitrogen for growth, because the quantity of nitrogen in the glutamate used and in the cells is of the same order of magnitude and the glutamate nitrogen appears as ammonia. During the later stage of growth of *Bordetella parapertussis*, some glutamate nitrogen does not appear as ammonia but this is not enough to account for the increase in cell nitrogen. It might be used to form arginine and ornithine, a reaction occurring in washed suspensions of this organism. Ammonia of the medium is not used up and no single amino acid is used in any quantity until after the glutamate of the medium has been exhausted. The cell nitrogen must, therefore, come from small quantities of many amino acids in the medium. It cannot yet be said whether these are degraded before incorporation or whether they are built into the cell protein unaltered.

Properties of the three species are summarized in Table 5.

Table 5. *Comparison of metabolism of* Bordetella pertussis, B. parapertussis *and* B. bronchiseptica

Organism	Mean generation time (hr.)	Main amino acid used	NH_3/Glutamate ratio in growth (moles)	Amino acids deaminated in growth on casein hydrolysate	NH_3/Glutamate ratio in washed suspension	Urease	Pigment formation	Glutamic decarboxylase
B. pertussis M 2400	2·8	Glutamate	1	Alanine, serine, glycine, aspartic acid, proline	0·76	—	—	—
B. parapertussis C 691	1·7	Glutamate	1–0·7	Alanine, serine, glycine, aspartic acid, proline, sometimes threonine	0·54	+	+	–
B. bronchiseptica 8752	1	Glutamate	1	All amino acids but arginine, lysine, histidine	0·62	+	–	+

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Proof of the Spontaneity of a Mutation to Penicillinase Production in *Bacillus cereus*

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SUMMARY: A stable mutation in a strain of *Bacillus cereus* from penicillin sensitivity to penicillin resistance due to the production of large amounts of penicillinase is reported. By the velvet pad replicate technique this mutation has been shown to be a spontaneous event occurring in the absence of penicillin. Penicillin-resistant mutants were obtained and the penicillinase production proved, without ever bringing the cells into contact with the antibiotic. The mutation rate is about 3×10^{-8} mutations per bacterium per division.

There is considerable argument over the respective importance of spontaneous mutation and specifically directed mutation or 'permanent adaptation' in bacteria. The example here described is important because it has been conclusively shown to be spontaneous, the mutation can be detected without treating the cells with the substrate, and the sensitivity of assay is great enough to detect a few molecules of enzyme/cell.

Most strains of *Bacillus cereus* produce penicillinase adaptively after pretreatment with penicillin for a few hours. Only one strain, out of 57 strains tested, failed to produce detectable enzyme even after such pretreatment; this was strain 5, which was isolated from soil (Knight & Proom, 1950; strain CN 2501). It is a typical strain of *B. cereus* differing from the majority of strains in not fermenting salicin, and in giving a delayed digestion of starch. Upon growth overnight in nutrient broth containing 1 unit penicillin/ml. the culture was found to have a high content of penicillinase; on plating out the culture most of the colonies were found to produce the enzyme in large amounts without pretreatment with penicillin. One colony was purified by plating and is the mutant 5/B.

METHODS

The organism was routinely grown upon tryptic meat digest broth, solidified when required by 1·5% Japanese agar and incubated at 30°. Spore suspensions were prepared by growth on agar slopes with the addition of 1 μg. manganous chloride/ml.; after a week the organisms were removed, washed three times with distilled water, sealed in ampoules, and after heating at 60° for 30 min., stored at 4°.

Tests for penicillinase activity. To the nutrient agar was added 4% of Andrade's indicator, and the addition of strong penicillin solution after growth of colonies gave red halos around penicillinase-producing colonies—the method described by Manson, Pollock & Tridgell (1954). A more sensitive test is to use agar containing 1% of starch; after growth the plates are flooded with 1%

iodine in 2% aqueous potassium iodide, the solution poured off, and strong penicillin solution (100,000 units/ml.) added. Colonies which produced penicillinase give pale halos. For strains which, unlike strain 5, digest starch rapidly this test is unsuitable, but colonies in agar without starch show pale halos on a brown background by this method.

For rough estimations of penicillinase in liquid cultures the quick assay of Perret (1954) was used with a calibration curve.

Penicillin. Crystalline sodium benzylpenicillin from Glaxo Laboratories Ltd., Greenford, Middlesex was used. Penicillin plates were made by adding freshly made solutions of the antibiotic to melted agar at 56°, and poured within a few minutes.

Plating technique. To avoid spreading of the organism in surface colonies and between the agar and the glass, sandwich layer plates were used, the bacteria being incorporated into the middle layer of medium. All three layers were of *c.* 15 ml. of tryptic meat nutrient agar with indicator.

Estimation of the proportion of mutants in a culture. The number of mutants in a culture could be estimated with reasonable accuracy, as determined by reconstruction experiments, by sandwich pour plates containing one of the penicillinase indicators, and 1 unit penicillin/ml., provided the total inoculum was not more than about 5×10^7 cells. Rather heavier inocula could be used when 2 units penicillin/ml. were added. At these concentrations of penicillin almost all the mutants form colonies and the parent cells fail to grow. Incubation was for 18 hr. at 30°. Only penicillinase-positive colonies were scored as mutants.

Velvet pad replicate technique. The technique devised by Lederberg & Lederberg (1952) was used with modifications as follows. Commercial velveteen with a short nap was cut into convenient squares, washed in distilled water and sterilized by autoclaving. A square was placed over the end of a waxed wooden cylinder 8 cm. in diameter, and held in place by an elastic band. Two sterile pins were placed in the velvet as markers of position. The block was soaked in hypochlorite solution (Chloros) and then rinsed in sterile water and dried with sterile absorbent cotton-wool before use.

Single spores of strain 5 were plated on nutrient agar. From this plate a single colony was spread on a nutrient agar 'master plate' to give a confluent growth, and after about 6 hr. of incubation this master plate was pressed on sterile velveteen (with pins for orientation), and was set aside at 4° until the next day. A number of penicillin agar plates (2 units/ml.) were then pressed in succession on the velveteen, and well dried. Since surface spreading of the growth was troublesome on the replica plates, it was necessary to touch in the imprints of the pins with sterile indian ink, to mark their position, and then to superimpose a layer of penicillin agar. This layer does not appear to disturb the position of the cells. These replica plates were incubated overnight. When resistant colonies were found at the same spots (relative to the pin marks) upon two or more of the plates, it was deduced that they were due to cells from a resistant micro-colony upon the master plate in the corresponding position. Two or three such areas of the master plate presumed to contain resistant clones (which must have arisen spontaneously since they had not been in contact with penicillin at any time) were cut out, and emulsified in broth. This broth was used to inoculate another master plate and the cycle repeated until a pure culture of mutants was obtained. The velveteen was used again after soaking in dilute Chloros (1/10) for 5 min., washing well in tap water and autoclaving.

RESULTS

Characteristics of the parent strain and the penicillinase-producing mutant strains

The parent and the mutant 5/B have the same colony form and biochemical behaviour apart from penicillinase production. No gross difference in growth rate was detected by inoculating equal numbers of viable spores of 5 and 5/B into the same tube of broth, and subculturing the mixed culture daily three times: the last broth culture still contained 50 % of mutants (within the limits of the counting error). The parent strain contains no penicillinase detectable by the quick assay of Perret (1954) or the manometric method of Henry & Housewright (1947), and is unable to produce it inductively; the mutant produces large amounts of penicillinase constitutively, and there is no appreciable increase when treated with penicillin. The sensitivity to penicillin is shown in Table 1. It is seen that single spores of the mutant are resistant to 4 units penicillin/ml., while spores of the parent are inhibited by 0·03 unit/ml.

Mutant 5/B has not lost its penicillinase-producing ability after many subcultures in penicillin-free media, and so far no penicillinase-negative colonies have been detected in subcultures. The mutant produces penicillinase, which has been crystallized, in an amount equivalent to 2 % of the dry weight of the cells (Pollock, Torriani & Tridgell, 1956). Penicillin-resistant variants which did not produce penicillinase were not found.

Table 1. *Penicillin sensitivity of* Bacillus cereus *strains 5 and 5/B when samples of about** 50 *spores were plated in penicillin nutrient agar*

	Penicillin concentration (units/ml.)											
	16	8	4	2	1	0·5	0·25	0·12	0·06	0·03	0·012	0
	Number of colonies developing in 18 hr. at 30°											
Parent (strain 5)	0	0	0	0	0	0	0	0	0	0	59	51
Mutant (strain 5/B)	0	20	42	57	50	50	64	72	55	59	61	66

* About 50 spores since actual number varied according to the usual random error.

Selection of mutants by growth in penicillin media

It was suspected that the enzyme-producing variants were of mutational origin when it was observed that plating of about 10^7 spores of the parent in penicillin plates (1 or 2 units/ml.) gave a constant number of resistant penicillinase-producing colonies, surrounded by satellite colonies of the parent. The latter evidently arose from organisms of the parent type which had survived until the variant colony had destroyed the penicillin in the immediate vicinity. Other parts of the plate were sterile or almost so after 24 hr. of incubation. When single spore clones of the parent strain were prepared, they also all gave mutants when plated in this fashion using inocula of 10^7 organisms/plate.

In penicillin broth (1 unit/ml.) it was observed that no growth occurred with small inocula of strain 5, while with larger inocula (*c.* 10^6 organisms) the culture which developed consisted almost entirely of mutants. With very large inocula the proportion of mutants after incubation was less, probably because the mutants destroyed the penicillin before all the parent organisms had been killed. There was considerable lysis of strain 5 in penicillin broth.

Experiments to demonstrate the occurrence of the mutation in the absence of penicillin

A number of techniques have been devised to decide whether mutations are induced specifically by the substance which causes selection of the mutants or whether they arise spontaneously, i.e. in the absence of the substance (Luria & Delbrück, 1943; Newcombe, 1949; Lederberg & Lederberg, 1952). As a preliminary test, the fluctuation test of Luria & Delbrück (1943) was used. One of the experiments is summarized in Table 2. It can be seen that, as required on the theory of spontaneous mutation, the variation between individual culture tubes was much greater than the expected counting errors.

Table 2. *Fluctuation test upon penicillin-resistant mutants of* Bacillus cereus

Twelve tubes containing 2 ml. samples of nutrient broth containing 30 viable spores/ml. of strain 5, were incubated for 18 hr. at 30°. The number of mutants in 0·1 ml. amounts was estimated by plating in penicillin agar (1 unit/ml.). Six replicate samples were plated from tubes 1 and 12; only one sample from each of the other tubes was plated.

Tube no.	Mutants in 0·1 ml. of broth	Total viable count/ml.
1	2 (replicate counts = 0, 2, 1, 2, 0, 1)	9×10^7
2	43	.
3	37	.
4	6	.
5	0	.
6	3	.
7	1	.
8	4	.
9	1	.
10	0	.
11	1	.
12	1 (replicate counts = 1, 2, 1, 3, 2, 0)	8×10^7

The approximate mutation rate can be estimated from the data. Using the second method of Lea & Coulson (1949) the median number of mutants/tube is 25, and m, the mean number of mutations/tube is 7·6. The mutation rate/bacterium/generation is 0·69 m/final population; in this experiment it was $3{\cdot}1 \times 10^{-8}$. Two further fluctuation tests gave estimates of $5{\cdot}6 \times 10^{-8}$ and $1{\cdot}8 \times 10^{-8}$. One may conclude that the mutation rate to penicillinase production is about 3×10^{-8}/bacterium/generation.

The constant proportion of mutants, about one in 2×10^5, in subcultures of strain 5 (provided the cells have reached a large number such as 10^{11}), demands explanation. The commonest reason given is that the mutation rate is balanced by a back-mutation rate, so that at equilibrium:

$$\text{Forward mutation rate} \times \text{proportion of non-mutants} \\ = \text{Backward mutation rate} \times \text{proportion of mutants}$$

In this case the formula would yield the figure of 6×10^{-3} for the back mutation rate, which is unusually high and which should have been easily detected by plating out of subcultures of mutants. No such back-mutants were detected. The more probable explanation is that the mutant cells have a slightly slower rate of growth upon the average than the parent cells. A small difference in growth rate will account for the equilibrium even in the absence of back mutation (Armitage, 1952). The relevant formula, in the absence of back mutation, is

$$\text{Proportional difference in growth rates} \\ = \lambda/\text{proportion of mutants at equilibrium.}$$

This is derived from Armitage (1952) as follows. The proportion of mutants at equilibrium $= g/(a+g-b)$ (from section 1·3); this is $(a+g)\lambda/(a+g-b)$, where $a+g$ is the growth rate of the non-mutant in Armitage's notation, and b the growth rate of the mutant (section 1·1). The proportional difference in growth rates $(a+g-b)/(a+g)$, equals $(g/(a+g)) \times 1$/proportion of mutants at equilibrium. The quantity $g/(a+g)$ is λ given above, and is the mutation rate expressed in equation 21 (Armitage, 1952), which is the mutation rate per bacterium per division cycle (as calculated above from the fluctuation tests) divided by 0·69 (see equation 20, Armitage, 1952). In the instance studied here, the mutants would have a difference in average growth rate of 6×10^{-3}, i.e. a growth rate 99·4% of that of the parent, which might not be detected in a short-term experiment.

Atwood, Schneider & Ryan (1951) also suggested that a major reason for the apparent stability of cultures of mutants and non-mutants is a constant mutation of the organisms in the majority to a form with a slightly higher growth rate, thus placing all the previous mutant organisms at a relative disadvantage, i.e. they are outgrown.

A number of objections have been made to the fluctuation test as a proof of spontaneous mutation; the most cogent is that of Dean & Hinshelwood (1952). They point out that where a culture of organisms which can adapt to a toxic substance is used, a very slight difference in the culture tubes or the plates used for counting mutants can give great fluctuation, since the number of colonies formed is only a reflexion of the number of cells which are able to become adapted before they are killed. This may depend on many uncontrollable factors. I have confirmed this in *Bacillus cereus* strain 569, which produces penicillinase adaptively; the number of colonies formed on penicillin plates fluctuates widely at certain critical levels of penicillin. It is therefore preferable to use another test for proof of spontaneity of mutations.

Use of the velvet pad replicate technique

This technique devised by Lederberg & Lederberg (1952) is the only one which gives unambiguous proof of the spontaneity of a mutation, since by this method one can isolate a mutant without ever exposing it to the selecting agent concerned. The test was performed successfully on three occasions, yielding each time a penicillinase-producing strain. On one occasion a distinctive colonial variant of strain 5 was used, which yielded a mutant of the same colonial type, thus excluding the possibility of chance contamination of the plates by a penicillinase-producing bacterium. The number of 'cycles' needed for isolation of a mutant was five or six. The degree of enrichment at each step is indicated by the percentage of mutants present in the broth used to inoculate the successive master plates in one series: 1st master plate, 0·00002 %; 2nd, 0·0004 %; 3rd, 0·01 %; 4th, 0·07 %; 5th, 0·1 %; 6th, 72 %; final culture, 100 %.

DISCUSSION

The velvet-pad technique, though published in 1952, has been little used for proving that mutations are spontaneous. There seem to be only four published examples of its use for this purpose: Lederberg & Lederberg (1952) showed that mutations to resistance to streptomycin and phage T_2 in *Escherichia coli* were spontaneous; Kraft & Braun (1955) found mutations causing antibiotic resistance to be spontaneous in *Brucella suis* and *Escherichia coli*; Goldstein (1954) showed that streptomycin resistant and dependent mutations occurred spontaneously in *E. coli*; Lam & Sevag (1955) used the method for studying the origin of streptomycin resistance. It is uncertain whether the latter authors fully understood the principles of this test; as they apparently only carried out a single 'cycle', it is most unlikely that the degree of enrichment would have given a population consisting mainly of resistant cells. Their failure to obtain resistant clones is therefore not unexpected.

There are only two formal objections to the technique: (1) that traces of antibiotic remain on the velveteen if it is used repeatedly, or in some other way the media become contaminated by traces of antibiotic; (2) the 'resistant' culture consists not of resistant organisms, but of organisms which are able to become resistant with great facility when they come into contact with the antibiotic. The latter behaviour could of course be due to selection either of a spontaneously arising variant with an exceedingly high rate of mutation which is specifically induced by the antibiotic, or of one composed of organisms which can all adapt to resistance by a mechanism similar to enzyme induction (for most antibiotics only a theoretical possibility). In the instance here described, the first objection is answered by the great lability of aqueous penicillin to heat, so that there were probably no traces in the velveteen or the media. The second objection is answered by the observation that Seitz filtrates of penicillin-free broth cultures of the three mutant clones contained large amounts of penicillinase; in view of the close correlation found between resistance and penicillinase production, it would seem reasonable to attribute the resistance to the presence of the enzyme. Therefore the agent responsible for resistance was produced in the absence of penicillin. It is not possible with most systems to demonstrate this since the organisms have to be treated with the antibiotic in order to prove that they are resistant.

It should be noted that absence of a positive result does not disprove spontaneity—it may be due, for instance, to slow growth of the mutant which nullifies the enrichment obtained in each step. At present, also, there is no way of estimating the number of mutations/plate because of the uncertainty of the amount of organisms transferred by the velveteen pad. The test is, however, so decisive when it is positive that it is well worth using despite its laboriousness. Once a change is proved to occur to some extent spontaneously, to prove specifically-directed mutation one must show that the antibiotic increases the rate of mutation, a problem beset with many technical difficulties as is well-known, but only too frequently ignored.

It should be noted that although the *Bacillus cereus* strain 5 is described here as penicillinase-negative, there is some evidence for a larger amount of enzyme in the cultures than is accounted for by the number of penicillinase-producing mutants present (Pollock & Sneath, to be published). This is being further investigated; the penicillinase content of the parent organisms must, however, be very small, of the order of one molecule/organism.

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Four New Diphasic Members of the Arizona Group of Bacteria found in Reptiles

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SUMMARY: Four hitherto undescribed diphasic members of the Arizona group of bacteria were identified in reptilian faeces. These organisms appear to occur frequently in healthy *Constrictor constrictor*.

Not many diphasic members of the Arizona group of bacteria have been recognized. This paper describes four new species found in the excreta of apparently healthy reptiles imported into England.

METHODS

All the organisms were isolated on deoxycholate citrate agar or Wilson and Blair agar, either inoculated directly or after enrichment in selenite broth. The methods used for biochemical and serological examination were those employed by Edwards, West & Bruner (1947) in whose paper the type cultures mentioned are described.

DESCRIPTIONS OF ORGANISMS

The organisms were motile rods with the usual appearances and cultural characters of enteric bacteria. All produced hydrogen sulphide, failed to form indole, were methyl red-positive and Voges–Proskauer-negative, utilized citrate and D-tartrate and did not hydrolyse urea. All fermented glucose, arabinose, xylose, rhamnose, maltose, trehalose, mannitol and sorbitol rapidly with the production of gas. Sucrose, adonitol, dulcitol, inositol and salicin were not fermented.

Organism 3829/53. From a tortoise (*Testudo graeca*) imported from Casablanca, Morocco: *Salmonella abortus-bovis* was found in the same specimen. This strain liquefied gelatin in 13 days and fermented lactose within 24 hr. It was agglutinated to titre by Arizona O group 9 antiserum and in adsorption tests removed all agglutinins from this serum. Of the H antigens phase 1 was identical with phase 1 of Pc. 195 (Arizona H 21) and phase 2 with phase 1 of Pc. 196 (Arizona H 26) (Edwards *et al.* 1947). The antigenic formula is therefore Arizona 9 : 21–26. Of these antigens only H 21, which is closely related to phase 2 of *S. chittagong* (z_{35}), is related to recognized Salmonella antigens.

Organism 3834/53. From a snake (*Constrictor constrictor*) imported from Mexico. The same specimen yielded the diphasic Arizona type 29 : 31–33. 3834/53 liquefied gelatin in 18 days and fermented lactose within 24 hr. The O antigens were identical with those of 557/52 (Arizona O 29) (Edwards, Kauffman & Fain, 1953). Phase 1 of the H antigens was closely related to phase 1 of *Salmonella poona* and identical with phase 1 of Cal. 1141 (Arizona H 31). Phase 2 removed all H agglutinins from *S. cholerae-suis* phase 1 serum and from Arizona H 32 serum. (The relations between this antigen and the Salmonella c antigen were described by Edwards, Kauffman & van Oye, 1952.) For the antigens found in both phases to be closely related to antigens found only in phase 1 in Salmonella is not uncommon in diphasic Arizona strains. The antigenic formula is 29 : 31–32.

Organism 599/54. From *Constrictor constrictor* from Mexico; this also harboured Arizona 29 : 31–33. Gelatin was liquefied in 15 days and lactose fermented after 4 days. This belonged to Arizona O group 16, the somatic antigens of which are identical with those of *Salmonella inverness*. When first examined this culture was poorly motile and was not flocculated by any H sera of the Arizona or Salmonella groups. Many passages through semi-solid medium were needed to induce motility, but ultimately a serum was produced which agglutinated the culture rapidly, completely and in high dilution. The organisms were cultivated in semi-solid medium to which 599/54 serum had been added in the hope of isolating a second phase but no migration occurred. The serum was then tested with all the H antigens known to occur in diphasic Arizona strains. Only H antigen 23 was flocculated and the reaction did not extend beyond a dilution of 1/1000. The O agglutinins and agglutinins for H 23 were removed from the 599/54 serum by adsorption with heated cells of the homologous culture and with phase 2 of Pc. 195 (Arizona H 23) (Edwards, *et al.* 1947). When this absorbed serum was added to semi-solid medium the culture spread rapidly through its substance. From the spreading growth organisms were obtained which possessed H antigens indistinguishable from those of Pc. 195, phase 2 (Arizona H 23). These antigens are related to but not identical with those of phase 1 of *S. uganda* (l, z_{13}). Since H antigen 23 has usually been found in combination with antigens which have been placed in the phase 2 of Arizona types, H 23 was designated as phase 1 in 599/54. The hitherto undescribed phase in which the culture occurred originally was designated phase 2 and given the symbol H 34. The formula is therefore Arizona 16: 23–34. An identical strain was isolated from another *Constrictor constrictor* imported at the same time, and shortly afterwards a third strain was received from the Michigan Department of Health. This had been isolated by Miss Elizabeth Cope at the autopsy of a snake of the same species at the Detroit zoo.

Organism 4601/54. From *Constrictor constrictor* from Mexico: Arizona 9 : 24–31, *Salmonella enteritidis* (*jena*) and *Salmonella* n.sp. 13, 22: z_{29} were found in the same specimen. This organism liquefied gelatin in 20 days and fermented lactose within 24 hr. The O antigens were not related to any of the known O antigens of the Arizona or Salmonella groups. Alcohol-treated suspensions of 4601/54 were not agglutinated by Arizona or salmonella O sera at a dilution of 1 in 50. Likewise an O serum prepared from 4601/54 which had a homologous titre of 1/6400 failed to agglutinate representatives of the various Arizona and Salmonella groups at a dilution of 1 in 50. The symbol 30 was assigned to this new O group. Of the H antigens phase 1 was agglutinated to titre by Arizona H 23 serum and in absorption tests reduced the titre from 1 in 5000 to 1 in 200. Phase 2 was agglutinated to titre by and removed all agglutinins from Arizona H 31 serum. The antigenic formula is Arizona 30 : 23–31.

COMMENT

For several years after they were first recognized diphasic members of the Arizona group were rarely found. There were two reasons for this. The majority of strains ferment lactose rapidly and are therefore discarded. The realization that the fermentation of lactose is not an absolute screening test *per se* will tend to correct this. The second reason is that so far reptiles have been the commonest source of these diphasic organisms, providing 19 out of a total 30 isolations. It is noteworthy that we found diphasic Arizona strains in the excreta of 8 out of 9 *Constrictor constrictor* examined. Besides those described we found the following: 9 : 24–31 (once), 16 : 23–25 (once), 29 : 31–33 (five times). Three snakes harboured two strains simultaneously. No monophasic Arizona strains were found. In contrast to this 311 examinations of the faeces from 45 specimens of *Testudo graeca* yielded only 3829/53 and 4 monophasic strains.

These findings add support to the supposition that the diphasic types of Arizona organisms tend to be less pathogenic than the more numerous monophasic types. On ten out of the nineteen occasions on which they have been found in reptiles they have been found at autopsy but their part in causing death is unknown. They have been isolated from the internal organs of turkeys in three flocks and in these instances no other cause of death could be found. Once they were found in the organs of foetuses delivered during an outbreak of abortion among ewes. In man they have been found twice: in a healthy food handler and in the faeces of a man with fever and diarrhoea.

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Genetic Recombination in *Pseudomonas aeruginosa*

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SUMMARY: Genetic recombination has been shown to occur in *Pseudomonas aeruginosa*. Crosses between 18 biochemical mutants of 4 strains were studied and, although non-random segregation was found for non-selective markers, it has not yet been possible to draw conclusions concerning gene order or linkage. The progeny obtained from crosses tended to resemble one parent more than the other. The 4 strains of *P. aeruginosa* used were interfertile in certain combinations but self-sterile. There was no evidence for an infectious principle affecting fertility but fertility changes may occur amongst recombinant progeny.

Genetic studies in *Escherichia coli* have demonstrated a mechanism of genetic exchange which until now has been unique among bacteria (see Lederberg *et al.*, 1951; Hayes, 1953). Even within this species practically all of the work has been restricted to the K12 strain. However, Cavalli & Heslot (1949) found that the strain NCTC 123 was fertile with K12, but its complex growth requirements made further work difficult. By means of a screening method Lederberg (1951) tested 2000 strains of *E. coli* and found over 50 to be cross-fertile with K12, and as far as was tested with each other. de Haan (1954) demonstrated recombination in *E. coli* strain B by transferring the F+ character from K12 to *E. coli* B. This strain had previously been thought to be infertile but is apparently F−. These results suggested that this type of genetic mechanism may occur not only in other strains of *E. coli* but also in other species of bacteria and for this reason genetic studies were begun with *Pseudomonas aeruginosa*. This organism grows luxuriantly on the usual bacteriological media, and grows adequately on a chemically defined medium of glucose and mineral salts. It is of low pathogenicity and can be readily isolated from a variety of sources.

METHODS

Bacterial strains

A collection of strains of *Pseudomonas aeruginosa* was obtained, and after studying their general cultural behaviour the following were selected for further investigation.

Strain 1, isolated from a wound, Melbourne. This strain freely produces green pigment and grows luxuriantly on nutrient agar. It gives a dense turbidity in nutrient broth with a thin surface pellicle and is lysogenic for a bacteriophage which attacks strain L.

Strain 3 was isolated from a wound at Melbourne. It grows readily on nutrient agar forming a scummy growth which does not readily emulsify and in nutrient broth it produces little turbidity but a well-developed pellicle.

Pigment is produced sparingly. The strain is naturally resistant to streptomycin and is lysogenic for a bacteriophage which attacks strains 1 and L.

Strain 29. This is a stock strain from the Department of Bacteriology, University of Adelaide. It produces pigment sparingly, grows readily on nutrient agar and produces a dense turbidity in nutrient broth with little pellicle formation. It is lysogenic for a phage attacking strain L.

Strain L. This is strain LIII-3bi isolated by Don & van den Ende (1950). It has a slower growth rate than the other three strains. In nutrient broth a poorly developed pellicle is formed. This strain does not produce pigment. It is sensitive to bacteriophages carried lysogenically by strains 1, 3 and 29.

Stock cultures were maintained on nutrient agar slopes at 4° from which subcultures to nutrient broth were made as required. A duplicate set of slopes was kept at −15°.

Media

Complete medium. Difco nutrient broth and agar with the addition of 0·5% Difco yeast extract. Pigment production on this medium was enhanced by the addition of 1% (w/v) glycerol.

Minimal medium. The medium ascribed to B. D. Davis by Lederberg (1950) was used. This is composed as follows: glucose, 1 g., dipotassium hydrogen phosphate, 7 g.; potassium dihydrogen phosphate, 2 g.; sodium citrate ($5H_2O$), 0·5 g.; magnesium sulphate ($7H_2O$), 0·1 g.; ammonium sulphate 1 g.; water 1 l.; agar (when required) 15 g.

Mutants

Nutritionally-deficient mutants for a variety of amino acids and purines were obtained by combination and appropriate modifications of the various techniques used by Demerec (1951), Davis (1948), Loutit (1952) and Lederberg & Lederberg (1952). The procedure was as follows.

The parent strain was inoculated into complete broth and grown with agitation at 37° for 24 hr. The culture obtained was centrifuged and resuspended in 0·3 M-sodium chloride for 60 min. at 37°. The suspension was centrifuged again and the organisms resuspended in 0·04% manganous chloride for 60 min. at 37°. After further centrifugation the packed organisms were inoculated into a fresh tube of nutrient broth which was then incubated with shaking at 37° for 18 hr. The bacteria from this growth were centrifuged, washed twice in saline, and finally suspended at a concentration of 10^7 organisms/ml. in minimal medium without ammonium sulphate and kept at 37° for 4–6 hr. without shaking. At the end of this period the suspension was centrifuged, the organisms resuspended to a concentration of 10^6/ml. in minimal medium + 15,000 units penicillin/ml. and incubated with shaking overnight at 37°. Sufficient penicillinase was then added to inactivate most of the penicillin, the culture being kept at 37° for 1 hr. to enable the enzyme action to take place. Dilutions of this culture (1/10, 1/100, 1/1000) were made in saline and 0·1 ml. samples of these dilutions plated on complete agar and incubated overnight. Selecting plates with a suitable colony density, replica plates on minimal agar were made, using the velveteen pad technique, and after overnight growth the replicas were compared with the nutrient plates. Any colonies which appeared on the nutrient plates but not on the minimal plates were subcultured and examined further. Identification of the specific growth requirements of the various mutants was made using the methods described by Lederberg (1950).

The frequency of recovery of mutants varied between 0·1 and 1% and differed from strain to strain. The various mutant requirements obtained are shown in Table 1. A number of mutants fell into the 'unknown' class, growth occurring with yeast extract but not with any single amino acid, vitamin, purine or pyrimidine, nor with any tested mixture of these substances. These mutants were not further investigated. The various mutants are referred to by the parent strain number or letter followed, in brackets, by the symbol for the biochemical requirement as given in Table 1.

Table 1. *Biochemical mutants obtained by treatment of* Pseudomonas aeruginosa *with manganous chloride*

Primary requirement	Growth also with	Symbol
Tryptophan	Indole	T_1^-
Tryptophan	.	T_2^-
Methionine	Homocysteine	M_1^-
Methionine	Homocysteine, cysteine, cystine	M_2^-
Arginine	.	A^-
Proline	.	P^-
Isoleucine + valine	.	IV_1^-, IV_2^-*
Leucine	Isoleucine	LIL^-
Lysine	.	L^-
Histidine	.	H^-
Threonine	.	Thr^-
Serine	.	Se^-
Adenine	Hypoxanthine	Ad^-

* For differences between IV_1^- and IV_2^- see Table 2.

Double biochemical mutants were obtained by the method described above with appropriate addition of the required supplement of the primary mutant. Mutants resistant to certain antibiotics were also obtained. Streptomycin-resistant mutants were obtained by plating heavy suspensions of bacteria on complete agar containing 250 μg. streptomycin/ml. Chloramphenicol-resistant mutants were obtained as single-step mutants by the gradient plate method of Szybalski & Bryson (1952). Gradient plates were prepared with the upper layer containing 250 μg. chloramphenicol/ml. A culture was then streaked over each plate which, after overnight incubation, showed the characteristic pattern of confluent growth on one side extending to discrete colonies on the side of greatest antibiotic concentration; these latter colonies were picked off as chloramphenicol-resistant mutants.

Recombinants were tested for resistance or sensitivity to these antibiotics by streaking them on nutrient agar and using commercially prepared compressed tablets (Sentests; Evans Medical Supplies Ltd.) containing the appropriate antibiotic or by streaking a culture of the recombinant on complete agar incorporating the antibiotic.

Crossing procedure

The method used for the detection of recombinants was that devised by Lederberg & Tatum (1946) in which the parents (auxotrophs) have different biochemical deficiency markers and selection is made for the nutritionally non-exacting recombinant progeny (prototrophs). The details of the procedure are as follows. Each parent strain was grown separately overnight at 37° on complete medium agar slopes. The growth was washed off in saline, centrifuged, washed twice in saline and then resuspended in saline to a concentration of 5×10^9 organisms/ml. Equal volumes of the two parent suspensions were then mixed and immediately plated out on the surface of a minimal agar plate in volumes of 0·2 ml./plate, the inoculum being spread uniformly over the surface of the plate with a bent glass rod. In all crosses a minimum of five plates of each parent alone was included as a control to detect and measure any back mutation to prototrophy.

To test recombinants further individual colonies were picked with a platinum needle and subcultured into tubes of liquid minimal medium. Such subcultures were slightly contaminated with the parent strains but not sufficiently to interfere with the tests for resistance to streptomycin or chloramphenicol. They were purified when required by streaking on nutrient agar and picking off single colonies.

RESULTS

A range of biochemical mutants was obtained for each of the four strains. Combinations of these mutants both within and between strains were chosen at random, and crosses made using the above procedure. It was found that with certain combinations prototrophic colonies appeared after 48 hr. while with other combinations no such colonies appeared; 72 hr. was subsequently taken as the incubation period for crosses and colony counts made after that length of time. It was found that some crosses produced more than 100 colonies/10^9 parental organisms, other combinations produced fewer colonies while some produced none at all. The number of crosses made was extended and the results of these crosses are shown in Table 2. As a working hypothesis, the formation of prototrophs under these circumstances can be taken to mean that some type of genetic re-assortment is taking place. The results from Table 2 indicate that not all combinations of the mutant strains used were fertile. All crosses between mutants derived from the one parental strain failed to produce prototrophs, i.e. the strains used were self sterile. Certain interstrain combinations, viz. 1 × L, 1 × 29, 1 × 3 were clearly fertile. The combination L × 29 has given variable results for three crosses and negative results for the rest, while the combinations L × 3 and 29 × 3 did not produce prototrophs in any of the tested combinations. Within any one of the fertile combinations, not all the crosses produced prototrophs. For example, with the combination 1 × L, $1(T_1^-)$ produced prototrophs when crossed to $L(IV_1^-)$, $L(LIL^-)$ and $L(Ad^-)$ but not when crossed to $L(Thr^-)$, $L(T_1^-)$ or $L(P^-)$. Thus the particular

Table 2. *Frequency of crossing between different strains and biochemical mutants of* Pseudomonas aeruginosa

	29(LIL$^-$)	29(Ad$^-$)	29(T_2^-)	3(M_1^-)	3(M$^-$)	L(P$^-$)	L(T_1^-)	L(Ad$^-$)	L(Thr$^-$)	L(LIL$^-$)	L(IV_1^-)	1(Ad$^-$)	1(IV_2^-)	1(IV_1^-)	1(LIL$^-$)	1(M_1^-)	1(T_1^-)	1(Se$^-$)
1(Se$^-$)	0	0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	
1(T_1^-)	0	0	+	+	+	0	0	+	0	+	+ + +	0	0	0	0	0		
1(M_1^-)	0	0	0	0	+	0	0	+	0	0	+	0	0	0	0			
1(LIL$^-$)	0	0	0	0	0	0	+	+ +	0	+	+ + + +	0	0	0				
1(IV_1^-)	0	0	0	0	0	+	0	+	0	0	0	0	0					
1(IV_2^-)	0	+ +	0	0	+ +	0	+	+	0	0	+	0						
1(Ad$^-$)	0	0	0	0	+	+	0	0	0	0	0							
L(IV_1^-)	0	0	0	0	0	0	0	0	0	0								
L(LIL$^-$)	0	0	0	0	0	0	0	0	0									
L(Thr$^-$)	0	0	0	0	0	0	0	0										
L(Ad$^-$)	0	0	0	0	0	0	0											
L(T_1^-)	+?	0	0	0	0	0												
L(P$^-$)	+?	+?	0	0	0													
3(A$^-$)	0	0	0	0														
3(M_1^-)	0	0	0															
29(T_2^-)	0	0																
29(Ad$^-$)	0																	
29(LIL$^-$)																		

+ + + + >125 recombinants/10^9 parental cells; + + +, 75–125; + +, 25–75; +, 5–25; +?, irregular results, $0<5$.

combination of biochemical mutant genes in any cross seems to determine whether or not prototroph formation will take place.

Double biochemical mutants were made for strains 1 and L. The primary mutants used were $1(T_1^-)$, $1(M_1^-)$, $1(LIL^-)$, $L(IV_1^-)$. The mutants obtained were $1(T_1^-LIL^-)$, $1(T_1^-A^-)$, $1(M_1^-H^-)$, $1(M_1^-T_1^-)$, $1(LIL^-T_1^-)$, $L(IV_1^-L^-)$, $L(IV_1^-Se^-)$, $L(IV_1^-T_1^-)$. Crosses were made between double mutants of strains 1 and L and in no case did the recombination frequency exceed 1 recombinant per 10^9 parent cells. For that reason double mutants have not been used to any great extent.

Assuming that factor recombination is occurring in *Pseudomonas aeruginosa*, then this could result from a fusion mechanism similar to that in *Escherichia coli*, by a transforming principle as in the pneumococcus, by an infectious process as in salmonella, or through some previously unencountered mechanism. For several reasons the fusion mechanism is thought to be the most likely.

Experiments similar to those of Zinder & Lederberg (1952) failed to produce or detect an FA principle. All experiments involving filtrates of cultures were in harmony with the conclusion that actual contact between the two parental organisms was needed for the formation of recombinants. The most critical of these experiments was a U-tube experiment as described by Davis (1950). The strains of *Pseudomonas aeruginosa* used in this experiment were $1(T_1^-)$ and $3(A^-)$, and were known to form prototroph recombinants when grown together in nutrient broth. The two parental cultures were placed separately, one in each arm, of a U-tube separated at the bottom by a Corning UF sintered glass filter. They were grown for 5–6 hr. at 37° with aeration. Then the culture medium was sucked from side to side through the sintered glass filter so that exchange of the culture fluids took place without mixing of the bacteria. This mixing of the culture fluids was continued for 4–5 hr. and then the culture incubated overnight. The cultures in each arm were then separately taken out, washed and plated. This experiment was repeated several times and in no case did recombination occur. As with *Escherichia coli*, this result suggests that actual contact of organisms was necessary for genetic recombination to take place.

The evidence against a transduction mechanism is supported by the fact that recombination for more than one genetic determinant occurred regularly. This was shown by studying the segregation of non-selective markers amongst the prototroph progeny. Two such markers were available, streptomycin resistance and chloramphenicol resistance. Accordingly, a number of crosses were examined, using those combinations of biochemical mutants and strains which gave sufficient numbers of prototrophs and observing the segregation of resistance and sensitivity to streptomycin and chloramphenicol amongst the prototroph progeny (Table 3). As it was necessary to obtain streptomycin-resistant mutants of a number of biochemical-mutant strains, the allelism of these independent mutations to resistance was tested by means of crosses 10, 11 and 12 in Table 3, and the results show that all the occurrences of S^r were allelic or else very close to one another. The results of crosses 1 to 9 indicate that the segregation of S occurred amongst the prototroph progeny and that the relative numbers of S^r and S^s among the progeny depended upon the particular selective biochemical markers of the parents. The segregation of C is unusual in that most of the progeny resembled one parent with respect to this character. In crosses of the type 1 × L and 1 × 3 the progeny resembled the strain 1 parent. In the one cross of the type 1 × 29 which was examined the progeny resembled the strain 29 parent in their reaction to chloramphenicol. Because of these results allelism tests for the various occurrences of C^r could not be carried out.

Table 3. *Segregation of S (streptomycin) and C (chloramphenicol) resistance in crosses between various biochemical mutants of* Pseudomonas aeruginosa

No.	Cross	S^rC^r	S^sC^r	S^rC^s	S^sC^s
1	$1(T_1{-}S^rC^r) \times L(IV_1{-}S^sC^s)$	138	174	3	0
2	$1(T_1{-}S^sC^s) \times L(IV_1{-}S^rC^r)$	0	0	105	67
3	$1(LIL{-}S^rC^r) \times L(IV_1{-}S^sC^s)$	260	23	6	0
4	$1(LIL{-}S^sC^s) \times L(IV_1{-}S^rC^r)$	0	1	12	126
5	$1(LIL{-}S^rC^r) \times L(T_1{-}S^sC^s)$	130	12	2	0
6	$1(T_1{-}S^rC^r) \times L(Ad{-}S^sC^s)$	72	96	0	0
7	$1(LIL{-}S^rC^r) \times L(Ad{-}S^sC^s)$	85	11	1	0
8	$1(T_1{-}S^sC^s) \times 3(A{-}S^rC^s)$	.	.	78	44
9	$1(T_1{-}S^rC^r) \times 29(T_2{-}S^sC^s)$	0	0	19	80
10	$1(T_1{-}S^rC^r) \times 3(A{-}S^rC^s)$	77	0	0	0
11	$1(T_1{-}S^rC^s) \times L(IV_1{-}S^rC^s)$	.	.	108	0
12	$1(LIL{-}S^rC^s) \times L(IV_1{-}S^rC^s)$	.	.	108	0

Table 4. *Segregation of S, C, M_1 and T_1 in progeny of the cross $1(M_1{-}T_1{-}S^sC^r) \times L(IV_1{-}S^rC^s)$ plated on minimal medium, minimal + tryptophan or minimal + methionine*

$1(M_1{-}T_1{-}S^sC^r) \times L(IV_1{-}S^rC^s)$

Medium	S^rC^r		S^rC^s		S^sC^r		S^sC^s		Total
Minimal	151		0		45		0		196
	M^+	M^-	M^+	M^-	M^+	M^-	M^+	M^-	
Methionine	68	183	5	2	24	181	2	0	465
	T^+	T^-	T^+	T^-	T^+	T^-	T^+	T^-	
Tryptophan	41	5	7	0	10	207	0	0	270

This lack of segregation has also been observed for the formation of pigment and for phage resistance. In crosses between strain 1 (pigmented, naturally resistant to phage 16 (Don & van den Ende, 1950) and strain L (non-pigmented, susceptible to phage 16) the progeny were almost invariably pigmented and of nearly 500 recombinant prototrophs tested from various crosses all were resistant to phage 16. The progeny of the cross $1(T_1{-}S^r)$ and $L(IV_1{-}S^s)$ were tested for their susceptibility or resistance to phages carried lysogenically by strains 1, 3 and 29 and for lysogenicity for strains 1, 3, 29 and L. Of 144 recombinants all were susceptible to a phage carried by strain 3 and were themselves lysogenic for a phage which acted on strain L. In other words they behaved like the strain 1 parent. It is quite clear, however, that in the crosses 1 to 9 of Table 3, S has segregated frequently and C infrequently to result in novel genotypes.

The segregation of non-selective biochemical markers was studied in the cross $1(M_1^-T_1^-C^rS^s) \times L(IV_1^-C^sS^r)$. The results of this cross are given in Table 4. It is clear that in minimal medium + methionine, M_1 has segregated to produce novel recombinants, and so has T_1 when the cross is plated on minimal medium + tryptophan. Lack of other suitable double-mutant stocks precluded further experiments of this type.

DISCUSSION

The described results lead to the conclusion that genetic recombination has occurred in *Pseudomonas aeruginosa.* The mechanism of this recombination is not clear however, and in view of the limited genetic data it would be unwise to speculate. It has not been possible to demonstrate any linear arrangement of the genes used. Even the allelism of certain biochemical markers is doubtful. The fertility patterns of the various mutants in the crosses shown in Table 2 have given some information concerning the allelic relationships of biochemical mutant genes used. For example, $1(IV_2^-)$ and $1(IV_1^-)$ have the same growth requirement, isoleucine + valine. Prototrophs are produced when $1(IV_2^-)$ is combined with $L(IV_1^-)$, $L(Ad^-)$, $L(T_1^-)$, $3(A^-)$ and $29(Ad^-)$, whereas $1(IV_1^-)$ produces prototrophs only when combined with $L(Ad^-)$ and $L(P^-)$ so that it is unlikely that the isoleucine-valineless genes of each of these strains are allelic. In addition, as the combination $1(IV_2^-) \times L(IV_1^-)$ produces prototrophs the two isoleucine-valineless genes involved are also probably not allelic. It is not possible to say whether $1(IV_1^-)$ and $L(IV_1^-)$ are in fact allelic, although the evidence is consistent with this being so. In much the same way $1(T_1^-)$ and $L(T_1^-)$ are probably allelic. They have the same growth requirements as each other (tryptophan, indole) and do not form prototrophs when crossed. It is curious, however, that the cross $1(T_1^-) \times L(IV_1^-)$ produced prototrophs freely, whereas the cross $L(T_1^-) \times 1(IV_1^-)$ failed to do so.

These uncertainties, together with the rather anomalous segregation in crosses involving $1(LIL^-)$ where the proportions of S^rC^r, S^sC^r, S^rC^s and S^sC^s were the same, whether $1(LIL^-)$ was crossed to $L(IV_1^-)$, $L(Ad^-)$, or $L(T_1^-)$, make it unwise to draw any firm conclusion about linkage and gene order at this stage. Before such conclusions could be made the segregation of more genes would have to be studied. However from the non-random segregation of S in the various crosses studied it becomes likely that some system of ordered genes is operating.

A search has been made in *Pseudomonas aeruginosa* for a factor similar to the F factor in *Escherichia coli*. *P. aeruginosa* clearly differs from *E. coli* in exhibiting self sterility. Such self sterility might indicate that strains were heterothallic and that the sexual differentiation is different from that of *E. coli.* It was found that the prototroph-forming capacity of *P. aeruginosa* was completely destroyed by streptomycin, in contrast to the situation with *E. coli* (Hayes, 1952). Experiments similar to those carried out by Cavalli, Lederberg & Lederberg (1953) in which the F^+ factor was readily transferred to F^- cells were repeated with *P. aeruginosa*, and yielded completely negative results. In short, fertility was not changed by any process of infection similar to that known to occur in *E. coli*. However, differences in recombination ability were found to occur amongst the pseudomonad recombinants. From the cross $1(M_1^-T_1^-S^sC^r) \times L(IV_1^-S^rC^s)$ plated on tryptophan, recombinants were picked off and certain T^- isolates were crossed to $1(LIL^-)$ and $L(IV_1^-)$. Of 9 isolates tested 5 crossed with $1(LIL^-)$ but not with $L(IV_1^-)$ and the other 4 crossed with $L(IV_1^-)$ but not with $1(LIL^-)$. The recombinants tested were all C^r and in two cases the crosses with $1(LIL^-)$ were analysed for segregation of C. In each case 96% of the progeny were C^s, i.e. like $1(LIL^-)$. This last result strongly suggests a change analogous to a change of polarity as occurs in *E. coli* (Hayes, 1953). Alternatively, perhaps, some factor or factors controlling the ability to cross with one or the other strain may be segregating.

Unexplained variations in recombination frequency have occurred during the course of these experiments, and preliminary studies have been made on factors affecting recombination frequency.

Certain strains have undergone spontaneous variation into various colonial types and the recombination frequency in certain tested crosses varies with these colonial types. The strain $L(IV_1^-S^r)$ gave rise to two colonial types designated $L(IV_1^-S^r)$t (transparent) and $L(IV_1^-S^r)$o (opaque). When each of these was crossed to $1(LIL^-)$ the recombination frequencies were $L(IV_1^-S^r)$t: 216, $L(IV_1^-S^r)$o: 4. The reason for such differences is not clear.

It has been found that the manner in which the cells are grown before being crossed affects the recombination frequency (recombinants/10^9 parental organisms). For the cross $1(T_1^-) \times L(IV_1^-)$ when the parental cells were grown under different conditions the recombination frequencies are as follows: (1) non-aerated broth, 30; (2) aerated broth, 51; (3) agar slopes, 146.

Other more subtle changes may take place. Cultures, which on first testing gave a certain recombination frequency, on subsequent testing in the usual manner failed to produce prototrophs at all, or in greatly diminished numbers. In some such cases crossing occurred when the parents were grown in aerated broth but not when grown on agar slopes. These changes have occurred in stocks kept at 4°. It will be recalled that the first detection by Hayes of the F^- condition in *Escherichia coli* was with a stock kept at 4°, and it is possible that the changes referred to earlier may be due to changes in such fertility factors. It is interesting to note that an F^- phenocopy was produced by Cavalli *et al.* (1953) when an F^+ culture was grown in aerated broth. So far it has not been possible to induce these changes in recombination frequency at will, and their occurrence has been too irregular to follow in detail.

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THE SOCIETY FOR GENERAL MICROBIOLOGY

The Society for General Microbiology held its Twentieth General Meeting in the Royal Institution, London, on Monday, Tuesday and Wednesday, 18, 19 *and* 20 *April* 1955. *The following communications were made:*

COMMUNICATIONS

Failure of *Chromobacterium violaceum* to Grow on Nutrient Agar, Attributed to Hydrogen Peroxide. By P. H. A. Sneath (*National Institute for Medical Research, London*)

It is well known that *Pasteurella pestis*, *P. septica* and *Shigella dysenteriae* may not grow aerobically on agar from small inocula, because they are highly sensitive to traces of hydrogen peroxide in the media.

Chromobacterium violaceum shows the same behaviour; of twelve strains, none grow on meat digest nutrient agar from small inocula. Heavy inocula grow well. The inhibition is abolished by blood, Fildes's digest, haematin (10 μg./ml.), manganese dioxide and by anaerobiosis. Protoporphyrin and starch are inactive. Non-pigmented variants also show the inhibition. Pure horse-liver catalase (0·01 μg./ml.) abolishes the inhibition, so the inhibitory substance is presumably hydrogen peroxide. The conclusion is supported by ultraviolet irradiation of the medium before inoculation, which increases the inhibition, while pretreatment with catalase (separated from the agar by cellophane and then removed) reduces it.

The organism has long been known to be exceedingly sensitive to sunlight (so that photographs can be printed on plate cultures) and it is very sensitive to ultraviolet light. Peroxide production may be partly responsible for both effects. The organism possesses catalase, but presumably single cells contain too little to protect them, a close parallel with *Pasteurella pestis*. *Chromobacterium violaceum* produces hydrogen cyanide, which may inhibit its own catalase.

Organisms with this behaviour are probably not uncommon. A diphtheroid has been found which behaves similarly to *Chromobacterium violaceum*. The phenomenon cannot be explained entirely by the extent to which bacteria produce catalase, since some organisms without catalase, such as streptococci, are commonly tolerant of peroxide.

The Measurement of Speeds of Motility in *Escherichia coli*. By R. C. Clowes, G. Furness and D. Rowley (*Wright-Fleming Institute of Microbiology, St Mary's Hospital Medical School, London*)

Various strains of *Escherichia coli* were found to differ in the times taken to spread across plates of sloppy (1 %) nutrient agar. By hanging drop, differences were observed in the rates of movement of the individual motile cells of different strains, and measurements of these speeds were made as follows.

Lengths (18 in.) of capillary tubing of 1 mm. bore are drawn out at one end which is silicone treated, and the other end joined to an inch length of 4 mm. bore soft glass tube. After heat sterilization, sterile 0·4 % molten nutrient agar is drawn up the tube so as to form an uninterrupted column of about 14 in. A 4 hr. broth culture of the motile strain, having previously been passed through a Craigie tube is then diluted (10^{-2}) and *c.* 1/50 ml. inoculated to make contact with one end of the solidified sloppy agar column, which is allowed to extrude slightly from the capillary into the wider end of the tube. The inoculum is now drawn into the capillary by allowing the agar to move towards the drawn out end, so that finally the agar column and inoculum occupy a position midway in the capillary.

The tube is then incubated horizontally, for 18 hr. at 37°. The drawn-out silicone end is sterilized by flaming, and by means of a perforated teat, the column extruded in measured lengths into aliquots of nutrient broth. The extent of movement of the motile organism down the capillary can then be found by the end-point of growth in the aliquots after incubation.

Escherichia coli 'B' controls showed no movement. No variations in movement were found with inocula which varied between no dilution and one of 10^{-3}, although decreases in movement were observed with increases of age of culture over 6 hours.

Using this technique, the characteristic movements of six mutants of the same *Escherichia coli* strain were found to vary from 4 to 11 in.

Potentiation and Destruction of Coagulase by Products of S. Variants of *Staphylococcus aureus*. By I. LOMINSKI, R. B. MORRISON AND D. D. SMITH (*Western Infirmary, University of Glasgow*)

A New Approach to the Study of Kinetics of Recombination in *Escherichia coli* K–12. By W. HAYES (*Postgraduate Medical School, University of London*)

The Influence of some Dicarboxylic Acids on Zygote Formation in *Escherichia coli* K–12. By K. W. FISHER (*Department of Bacteriology, Postgraduate Medical School, University of London*)

An investigation of the conditions controlling zygote formation between auxotrophic strains of *Escherichia coli* K–12 using principally M–V_6^sHfr and (TLB_1)–V_6^rF– has been started.

Zygotes are allowed to form by aerating at 37° a mixture of washed parent strains, and at the end of 30 min. 1 ml. of the mixture is added to 1 ml. of coli phage T_6 (10^{10} particles/ml.). This destroys the Hfr parent and stops further recombination. Zygotes already formed can be detected by diluting the mixture and plating on minimal agar supplemented with B_1.

Very little recombination occurs when the strains are aerated together at 37° in M/20 phosphate buffer (pH 7·2) for 30 min.—in nutrient broth under the same conditions a high rate of recombination is observed.

This high rate can be achieved if the buffer be simultaneously supplemented with glucose (20 μmole/ml.) and L-aspartic acid (100 μg./ml.). The actual number of prototrophs formed depends on the concentration of aspartic acid—the optimal being at about 100 μg./ml.

Stimulation to a lesser degree can be achieved with succinic, fumaric and malic acids, together with glucose if the mixture is buffered at pH 6·5. The increased rate can be reversed by malonic acid (6·5 mg./ml.).

No effect on recombination is observed when NH_4^+ (50 μg. ammonia-N/ml.) is added to the system either alone or in combination with glucose thus ruling out the possibility that the stimulation of recombination by L-aspartic acid may be due to the α amino nitrogen. The experimental results do not allow a differentiation between the possibilities of L-aspartic acid acting (*a*) as an adsorption cofactor, or (*b*) via the intermediary of the Krebs cycle.

Differences in Cell-wall Composition among Gram-positive Cocci and Bacilli.

By C. S. Cummins and H. Harris (*Departments of Bacteriology and Biochemistry, The London Hospital Medical College*)

Cell-wall fractions prepared by shaking bacteria in a Mickle tissue disintegrator were separated by centrifugation and purified by digestion with crystalline trypsin and ribonuclease, followed by crystalline pepsin. Electron microscopy of a few preparations indicated that the method gave adequately pure material. The purified cell walls were hydrolysed and examined by paper chromatography for amino acids and sugars.

Eight species of Corynebacteria showed large amounts of arabinose, galactose, alanine, glutamic acid and $\alpha\epsilon$-diaminopimelic acid; smaller amounts of glucose and/or mannose; and only traces of other amino acids.

Streptococci of Lancefield's groups A–G showed large amounts of rhamnose, alanine, glutamic acid and lysine. Except in group A, one or more of the hexoses glucose, galactose and mannose also occurred. Arabinose and diaminopimelic acid were absent.

Six strains of *Corynebacterium pyogenes* contained no arabinose or diaminopimelic acid, but closely resembled Streptococci in showing rhamnose, alanine, glutamic acid and lysine, with smaller amounts of glucose and mannose.

Staphylococci showed large amounts of alanine, glutamic acid, lysine and glycine. Rhamnose, arabinose and diaminopimelic acid were absent.

Four strains of *Aerococcus viridans* (Williams, Hirch & Cowan, 1953) showed the streptococcal amino acid pattern, but lacked rhamnose.

A few species of Lactobacilli and Bacilli appeared similarly to show distinctive patterns of cell-wall constituents.

All species examined contained glucosamine and an unidentified hexosamine-like substance similar to that described by Strange & Powell (1954). Chondrosamine was present in some species but absent from others.

The results indicate the possibility of differentiating bacterial genera, and perhaps species, by their cell-wall composition, and suggest that

Corynebacterium pyogenes should be reclassified and placed in the genus *Streptococcus.*

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Protein, Nucleic Acid and Adaptive Enzyme Formation in Protoplasts of *Bacillus megaterium*. By K. McQuillen (*Department of Biochemistry, University of Cambridge*)

Treatment of *Bacillus megaterium* with lysozyme causes dissolution of the cell walls and cross-septa, and if carried out under controlled conditions in sucrose media, results in the formation of two, three or four spherical protoplasts from each original rod-shaped organism (Weibull, 1953). These subcellular preparations do not form colonies and have not been shown to be able to divide, but they are capable of carrying out a wide range of synthetic reactions. Isotopic tracers (glucose, glycine, uracil, thymine and acetate) have been used to demonstrate amino acid and protein formation and nucleic acid synthesis.

Glucose provides carbon for lipids, nucleic acids and protein; glycine gives rise to protein glycine and also adenine and guanine of the nucleic acids; uracil labels uridylic and cytidylic acids of the nucleic acid fraction; thymine is not used even by intact living *B. megaterium*. Acetate carbon becomes incorporated in the lipid fraction and also in the pyrimidines—probably via aspartic acid; it also appears in glutamic acid, aspartic acid, threonine, lysine, arginine, proline, leucine(s) and alanine of the protein (this is very similar to the behaviour in *Escherichia coli* where the tricarboxylic acid cycle provides intermediates for amino acid synthesis—McQuillen & Roberts, 1954). Intact *Bacillus megaterium* contain and synthesize diaminopimelic acid under the conditions used: protoplasts do neither.

Protoplasts are also able to synthesize the adaptive enzyme β-galactosidase.

The rates of all these processes in protoplasts are comparable with the rates in intact growing cells.

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The Formation of Spores in Protoplasts of *Bacillus megaterium*. By M. R. J. Salton (*Department of Biochemistry, University of Cambridge*)

Protoplasts of *Bacillus megaterium* formed by the dissolution of the cell wall on treatment with lysozyme in a sucrose medium do not give rise to colonies when plated on media supporting the growth of the intact organism. Although it seems entirely possible that protoplasts may possess the ability to produce colonies under very special growth conditions, an alternative mechanism of survival by formation of intra-protoplast spores appeared conceivable.

Investigations by Hardwick & Foster (1952) have suggested that sporogenesis is an intracellular process and that certain spore-forming Bacillus species could be induced to spore on shaking in distilled water. Several strains of *Bacillus megaterium* used in the present study have indeed given rise to as many as 90% spores when shaken in distilled water. However, sporulation of these strains in distilled water was found to vary widely from day to day experiments, and the percentage cells containing intracellular spores varied from 0 to 90%. More consistent results were obtained when sporulation was carried out in 7·5% (w/v) sucrose in M/30 phosphate buffer (pH 7), and the addition of M/100 Mg^{++} to this medium always gave higher percentage sporulation values.

Protoplast experiments have been performed after preliminary shaking of the vegetative cells in distilled water or in the sucrose-phosphate medium with and without Mg^{++} (M/100). The ability of protoplasts to give rise to mature bacterial spores (which in turn can germinate and produce colonies when plated on a 5% peptone, 0·1% starch agar medium) has been followed by estimating the numbers of viable spores. In a typical experiment intact cells gave 10^7 viable spores/ml., protoplasts 10^5 viable spores/ml., whereas the number of viable spores in the lysed protoplast preparations remained unchanged at the low initial level of 10/ml. The capabilities of the protoplasts to produce mature intra-protoplast spores have been further confirmed by examination in the phase-contrast microscope—the highly refractile spore may be seen inside the protoplast membrane.

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Specific Di- and Tri-nucleotides as Activators of Amino Acid Incorporation in Disrupted Staphylococcal Cells. By E. F. Gale (*Medical Research Council Unit for Chemical Microbiology, Department of Biochemistry, University of Cambridge*)

Effects of Amino Acids on the Production of Transaminases by *Neurospora crassa*. By J. R. S. Fincham and A. B. Boulter (*University College, Leicester*)

Transamination was studied between L-amino acids and α-ketoglutarate, glutamate formation being determined. Extracts of *Neurospora crassa* mycelium grown on minimal medium showed relatively high aspartic acid and alanine transaminase activities (the latter showing a marked requirement for pyridoxal phosphate), and lower ornithine transaminase activity. Other transaminase activities were generally low. Supplementation of the growth medium with valine, leucine, isoleucine, phenylalanine or methionine resulted in an increase in ornithine transaminase activity of the order of 2- to 4-fold, but a much more striking stimulation (about 10- to 20-fold) of nine transaminase activities which were generally low in mycelium grown on minimal medium. These nine activities varied from one preparation to another in a parallel fashion and showed consistently the following decreasing order: phenylalanine, isoleucine, valine, leucine, norleucine, norvaline, methionine, α-aminobutyric acid, tryptophan. It is suggested that all these activities may be the property of a single enzyme. Aspartic acid, alanine and ornithine transaminase activities appear to be due to three specific enzymes.

Hydrogen-oxidation and Phosphorylation in Micro-organisms.

By H. G. Schlegel (*Institut für Kulturpflanzenforschung in Gatersleben der Deutschen Akademie der Wissenschaften zu Berlin*)

The oxidation of molecular hydrogen by autotrophically grown resting cells of *Hydrogenomonas* has been shown to be coupled with the uptake of inorganic phosphate (Schlegel, 1954). A great deal of this phosphate is recovered in the fraction which is split by boiling with 1 N-HCl for 7 min. Only a small part of this fraction is TCA-soluble, the main part remains behind in the cells during the TCA-extraction and seems to be polymetaphosphate. The oxidation of organic H-donators like succinic acid, pyruvic acid or glucose is equally coupled with phosphorylating processes, though more phosphate is recovered in other fractions. Maximum results were obtained with cells which were deprived of stored material and of energy-rich phosphates by aeration and successive anaerobic incubation. The separation in time of phosphorylation and CO_2-fixation was not possible. For phosphorylation the presence of CO_2 proved to be a *conditio sine qua non.* When CO_2 was absorbed by alkali, dephosphorylation prevailed even during the oxidation of hydrogen. It may be concluded that respiration cannot be substituted by hydrogen-oxidation. The coupling of oxidation of organic acids with phosphorylation is also dependent on the presence of CO_2. The heterotrophic metabolism of hydrogen-oxidizing bacteria seems to be closely related to the type occurring in the metabolism of the Athiorhodaceae.

As our experiments were done under conditions similar to those of Vogler & Umbreit (1942) in their investigation on the separation of thio-oxidation and CO_2-fixation, we may conclude that in the phosphorylation of the hydrogen-oxidizing bacteria there is involved a mechanism other than that in *Thiobacillus thiooxidans.*

New results were obtained with fresh cells, which were rich in stored material and energy-rich phosphates. When these suspensions are shaken in air, the amount of inorganic phosphate remains relatively constant for several hours. But if these cell-suspensions are shaken in bottles, which contain alkali in the centre-well, the amount of inorganic phosphate increases, mainly at the expense of the energy-rich phosphate-fraction. It is evident that CO_2 plays a decisive role even in the utilization of the energy released during the oxidation of the cell's own material. CO_2 seems to function not only as a substrate or as a product of metabolic processes but has the function of an important intermediary metabolite. In experiments with *Rhodospirillum rubrum* and *Chlorella pyrenoidosa* similar results were obtained. However, the phosphorus metabolism of yeast was not appreciably influenced by the presence or absence of CO_2. Whether or not phosphorus metabolism can be regarded as an indicator of the utilization of CO_2 must be explored in further investigations.

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Effects of 2-Heptyl 4-Hydroxyquinoline-N-oxide (a Streptomycin Antagonist) on Cell-free Preparations of *Escherichia coli*.

BY F. L. JACKSON (*King's College Hospital Medical School, London*) AND J. W. LIGHTBOWN (*National Institute for Medical Research, London*)

Previous communications (Lightbown, 1954; Lightbown & Jackson, 1954), have described the antistreptomycin activity of 2-heptyl 4-hydroxyquinoline-N-oxide, and some of its effects on the cytochrome systems of heart muscle succinic oxidase and *Staphylococcus aureus*.

Although the antagonist was highly effective in counteracting the inhibition of *Staphylococcus aureus* and *Bacillus subtilis* by streptomycin, it was practically without effect with *Escherichia coli*. This was paralleled by absence of inhibition of oxygen uptake by washed suspensions of *E. coli* with added succinate and lack of effect on cytochrome oxidation and reduction as observed spectroscopically. Antagonist was strongly absorbed from solution by whole cell suspensions of *Staphylococcus aureus* and *Bacillus subtilis*, but suspensions of *Escherichia coli* absorbed none. It seemed possible, therefore, that the antagonist might be unable to penetrate the cells.

Escherichia coli cells were broken by means of a Mickle disintegrator, the debris removed by centrifugation at 800 *g*. and the practically cell-free supernatant fluid collected. This had strong succinic oxidase activity and showed spectroscopically the bands of cytochromes b_1 and a_2. Antagonist produced up to 50% inhibition of O_2 uptake with succinate by this preparation and spectroscopic examination showed that there was a two- to threefold increase of the reduction time of oxidized cytochrome b_1. This contrasted with *Staphyloccus aureus* and heart muscle, in which oxidation of reduced cytochrome b components was inhibited, and it seems possible that the b_1 of *Escherichia coli* might differ functionally from these. Furthermore, the materials removable from the preparations by centrifugation at 17,000 *g*. strongly absorbed antagonist from solution (unlike whole *E. coli* cells), and the absorbed antagonist could be recovered.

Preparations of *Proteus vulgaris* behaved in a similar manner to those of *Escherichia coli*. The failure of the antagonist to penetrate the cells of the two Gram-negative organisms examined might account for the lack of antistreptomycin activity in these cases.

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The Relationship between Growth Rate of Different Bacteria and the Formation of Antibiotic Inhibition Zones. By A. H. LINTON (*Department of Bacteriology, University of Bristol*)

By preincubation experiments, it has been shown that the position of antibiotic inhibition zones in agar-diffusion assays is fixed at a critical time following commencement of incubation of the test organism. This time (T_0) is the sum of the lag (L) plus a definite number (n') of generations (G) of the organism, thus

$$T_0 = L + n'G.$$

Factors influencing lag or growth rate (e.g. temperature) correspondingly alter the value of T_0.

When the inoculum size was kept constant, the number of generations developing in time T_0 remained constant over a wide temperature range. For *Staphylococcus aureus* var. Mayo and either streptomycin or chloramphenicol, the calculated 'n'' value was 4·25.

Further evidence was obtained by counting the number of organisms in the colonies just inside the inhibition zone, in stained preparations of seeded agar-diffusion columns incubated either for 24 hr. or slightly longer than the T_0 period. In both cases the mean value was twenty-six organisms per colony—a count which falls between four and five generations. These findings demonstrate that a critical density of organisms develops at the zone edge in time T_0. The n' value for *Bacterium coli* and streptomycin was 4·0, being slightly lower than for the staphylococcus.

When the inoculum size was varied, T_0 correspondingly varied, indicating a relative increase or decrease in the number of generations required to reach the same critical density.

These results suggest that up to time T_0, the concentration of antibiotic diffusing into the agar is sufficiently high to inhibit the test organism. Once, however, a critical density of organisms is reached at time T_0, the subsequent logarithmic increase in growth is too great to be inhibited by the exponential increase in antibiotic concentration at the zone edge, and hence the colonies increase in size making the edge macroscopically visible.

Stimulation in Mice of Natural Immunity to *Escherichia coli* Infection. By D. ROWLEY (*Wright-Fleming Institute of Microbiology, London*)

Studies on the Distribution of Isoniazid-resistant Tubercle Bacilli within the Lung. By SHEILA M. STEWART AND F. W. A. TURNBULL (*Southfield Hospital, Edinburgh*)

The Formation of Hyaluronidase during Continuous Culture of Staphylococci. By H. J. ROGERS (*National Institute for Medical Research, Mill Hill, London*)

In shaken broth cultures of four strains of *Staphylococcus aureus* the appearance of hyaluronidase in the culture fluid at first lags behind growth and then increases more rapidly than bacterial dry weight: during this time growth of the organism is occurring logarithmically. Enzyme formation ceases after the logarithmic growth phase but before the stationary phase is reached. If the logarithmic phase is prolonged by growing the organisms in a 'chemostat' or 'bactogen' at or near their maximum growth rate and at constant opacity, the hyaluronidase activity in the culture fluid, and hence the ratio of enzymic activity to mass of growth (E/G), increases in a logarithmic fashion. If an 18 hr. shaken culture is diluted so as to give *c.* 10^6 cells/ml. of fresh medium and the culture then grown to a given density and maintained there in the bactogen, the increase in E/G continues for eleven generations, irrespective of the mean generation time of the organisms which varied from 21 to 40 min. in different experiments. Thereafter E/G remains constant, and if these cells are removed to a fresh medium growth continues with a constant E/G ratio. If, however, such a culture growing with a constant E/G ratio is allowed to grow for 18 hr., i.e. into the stationary phase of hyaluronidase formation, the cells when inoculated into fresh medium again show a lag in enzyme formation followed by a phase of increasing E/G ratio.

These observations are consistent with a change in the cells during the later stages of growth in shaken culture such that they either become deficient in a factor or factors necessary for hyaluronidase formation but not for growth or accumulate an inhibitor of enzyme formation.

The Effect of Fibrinogen on certain Pathogenic Cocci. By E. S. Duthie and E. Ball (*Royal South Hants Hospital, Southampton*)

Hyaluronidase Production by Group B Streptococci and by other Bacteria associated with Bovine Mastitis. By D. G. Howell (*A.R.C. Field Station, Compton, Berks*)

An investigation into hyaluronidase production by 170 strains of Lancefield group B streptococci obtained from both human and bovine sources showed that the latter were consistently high producers, whereas very few strains of the former produced this enzyme. It was also found that the majority of typeable high producing strains occurred within Lancefield precipitin Type II. A correlation also existed between the manifestation of protein antigens of the 'R' kind and the ability to produce hyaluronidase. Furthermore, those strains which failed to type within the Lancefield schema, i.e. those lacking a type polysaccharide and which could be classified by the protein antigens only, were usually high hyaluronidase producers.

Hyaluronidase was also produced by all strains of *Staphylococcus aureus*, *Streptococcus dysgalactiae* and by a minority of strains of *S. uberis* obtained from cases of mastitis. *Corynebacterium pyogenes* associated with cases of summer mastitis did not produce the enzyme. It should be noted, however, that the role of this organism as a prime pathogen is somewhat doubtful as it is usual to find one of the pathogenic mastitis streptococci in association.

In order that the disease process can be initiated, it is necessary for the epithelium of the ducts or secretory acini of the mammary gland to be penetrated. Of the extracellular products of the group of organisms associated with infectious bovine mastitis, hyaluronidase appears to be a distinct possibility as the mechanism by which such penetration could be effected. Experiments have been carried out using *Mycobacterium phlei*, cultures of which have been injected into goat mammary glands with and without purified hyaluronidase. Penetration of both ducts and acini have been observed in histological section when hyaluronidase was added to the inoculum but not in its absence.

Different Components of the Anthrax Toxin; their Relationship to Protective Antigens.

By H. Smith, D. W. Tempest, J. L. Stanley, P. W. Harris-Smith and R. C. Gallop (*Microbiological Research Department, Porton, Wilts*)

The specific, oedema producing, lethal factor in plasma of guinea-pigs dying of anthrax (Smith & Keppie, 1954) is serologically connected with a protective antigen of *Bacillus anthracis* produced *in vitro* by Strange and Belton (1954). Smith & Gallop (unpublished) obtained from anthrax exudates an essentially homogeneous protective antigen which was deposited by ultracentrifugation (100,000*g*) for 6 hr.

Similar ultracentrifugation of the toxic plasma produced a supernatant and a deposit. The deposit was washed and dissolved in saline. These two components caused only small skin reactions and had negligible lethal activity if injected separately. When injected together they formed a synergic mixture. Thus, skin lesions produced in guinea-pigs by the mixture were greater than those formed by each component at a concentration four times that in the mixture. In tests with groups of five to ten mice, intravenous injection of the mixture killed all animals, whereas the separate components killed no animals even when concentrated fourfold.

The deposit contained *c.* 60% of an ultracentrifugal component corresponding to the protective antigen from anthrax exudates.

The supernatant factor could not be replaced by normal plasma, its own ultrafiltrate, polyglutamic acid or extracts of *Bacillus anthracis*. The supernatant induced active immunity and its replacement by the protective antigen (0·1%) from *in vitro* culture, produced identical results in the mouse and skin tests described above. However, this antigen and the supernatant factor are not identical since a mixture of them also produced a synergic effect in the mouse test; this effect was specifically neutralized by antiserum and not shown in skin tests.

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Studies on Intracerebral Typhoid Infection in Mice.

By M. Landy, S. Gaines and H. Sprinz (*Army Medical Service Graduate School and Walter Reed Army Hospital, Washington, D.C.*)

In potency tests of typhoid vaccine, mice are injected intraperitoneally with *Salmonella typhosa*. Unprotected animals show symptoms of acute intoxication shortly thereafter, generally succumbing within 36 hr. Certain aspects of this procedure, such as magnitude of challenge dose required, or employment of mucin, and the overwhelmingly toxic death which ensues, have resulted in criticism of conclusions based on its use. Intracerebral inoculation avoids these objectionable features. In seventeen inbred strains of mice, susceptibility to this challenge varied from 18 to 100 % mortality. Only those typhosa strains rich in Vi and O antigens were highly virulent ($LD_{50} < 200$ cells). In the most susceptible mice (BALB/c) inoculated with 5000–40,000 *S. typhosa* Ty 2, few deaths occurred during the first 2–3 days, although most animals appeared ill. By the 4th–6th day, animals generally exhibited paralysis and rapidly succumbed, mortality reaching 100 % by the 7th–10th day. Significant, but not extensive multiplication occurred in the brain within 12 hr. after challenge, increasing rapidly after 18–24 hr. and attaining a maximum of 0·1–1·0 billion organisms, which was maintained until death. Bacilli were also found in heart's blood, spleen, and liver at 12 hr., these organs yielding 1,000–100,000 organisms until death. Histologically, an acute meningitis is discernible 12 hr. after inoculation. At 24 hr. there is definite evidence of meningo-encephalitis, and by 36 hr. this lesion is fully established, showing early suppuration, and changing thereafter only in intensity. Thus, intracerebral typhoid infection in mice may be a useful experimental technique which, in contrast to the intraperitoneal procedure, requires only small numbers of organisms without adjuvant, and provides a progressively developing fatal infection, exhibiting a well-defined incubation period, characteristic bacterial dissemination and brain pathology.

The Pectic Enzymes of Bacterial Pathogens of Plants. By W. K. Smith

(*Akers Research Laboratories, Welwyn*)

As a basis for the development of specific enzyme inhibitors for the treatment of plant diseases the ability of pathogenic, and other, bacteria to produce pectic enzymes was examined.

Seventy-six cultures representing 31 species in 9 genera were tested for ability to produce α-, β, -or γ-pectinglycosidase (P.G.) and 52 strains (27 spp., 6 genera) for pectin methyl esterase (P.M.E.) production.

Of the pectinglycosidases only γ-P.G. (optimum $pH > 7{\cdot}0$) was detected. Positive results were obtained from the following genera: *Erwinia* (13 out of 14 strains), *Pseudomonas* (5 out of 15), *Xanthomonas* (3 out of 16), *Bacillus* (5 out of 6), coliform group (1 out of 14), *Flavobacterium* (1 out of 1). Four strains of *Agrobacter*, 2 of *Corynebacterium* and 4 of *Rhizobium* gave negative results.

Ability to produce P.M.E. was restricted to the soft rot bacteria (*Erwinia carotovora, E. aroideae, E. atroseptica* and *E. phytophthora*) and *Xanthomonas campestris*. All these bacteria have been reported to cause vascular browning in infected plants.

An Interference Phenomenon caused by *Pasteurella pestis*. By W. R. Parry (*Department of Pathology, St Helier Hospital, Surrey*)

Small white laboratory rats were injected intraperitoneally with buffer dilutions of broth cultures of *Pasteurella pestis*. The cultures were grown at 28° with constant shaking. Challenge doses of 10^6 organisms of strain L37 killed rats less quickly than 10^4 organisms. Similarly, 10^6 organisms of strain I72 killed fewer rats than 10^4 organisms.

It has been stated that a culture of virulent *P. pestis* consists of a mixture of organisms varying from fully virulent to completely avirulent. The number of avirulent organisms in the challenge dose was therefore increased. $3{\cdot}8 \times 10^7$ organisms of Otten's vaccine strain Tjiwidej, when added to 10^4 strain L37, gave complete protection to the rats.

10^6 organisms of strain L37, killed by either ultraviolet light or by exposure to 59° for 30 min., afforded some degree of protection against 10^4 viable L37 organisms injected at the same time. This protection was less than that given by 10^6 viable organisms of strain L37.

Intravenous injection of indian ink, prior to an intraperitoneal challenge of $6{\cdot}4 \times 10^3$ L37, prevented the death of six out of eight rats.

No increase in the bactericidal action of the rat's serum was detectable after challenge with an interfering dose. The phenomenon is probably due to a non-specific stimulation of the defences of the rat.

An Experiment to Test a Theory concerning the Significance of the LD_{50} in Infective Systems. By G. G. Meynell (*Postgraduate School of Medicine, University of London*) and B. A. D. Stocker (*Lister Institute of Preventive Medicine, London*)

Mice were challenged by intraperitoneal injection with a suspension containing equal numbers of three variants of *Salmonella paratyphi* B. The variants possessed the flagellar antigens *b*, *i* and *eh* respectively, and their LD_{50} doses were not significantly different, being 10^7 bacilli. Heart blood was removed from each mouse *post-mortem* and plated to give single colonies which were scored by slide agglutination. Most of the mice dying from a dose of 0·5–0·25 LD_{50} and a minority of those dying from 1 LD_{50} yielded 80–100 % of only one variant. Each variant was found with approximately the same frequency in this way.

It is considered that these results are incompatible with any theory of infection which assumes that all or a substantial fraction of the inoculated organisms multiply and contribute to the fatal infection. (Theory of dependent action.) The simplest of such theories is that which postulates the existence of a minimal lethal dose for each individual host. The observations are partially consistent with a theory of independent action which assumes that there is a small probability of each inoculated organism causing a lethal infection in a given host, and that the fate of each organism is independent of that of any other inoculated organism. Two observations were inconsistent with the predictions of this theory: few completely pure infections occurred, and too many mixed infections were found in mice dying from a dose of 1 or >1 LD_{50}. These discrepancies are tentatively attributed to a terminal decrease in resistance enabling organisms to multiply freely which formerly were restrained. We therefore regard the LD_{50} in this system as that dose at which there is a probability of 0·5 that at least one inoculated organism will give rise to a fatal infection.

Effect of Growth Medium on Oxidative Activity in *Saccharomyces cerevisiae*. By C. F. STRITTMATTER (*Microbiology Unit, Department of Biochemistry, University of Oxford*)

After adaptation to growth on 2% galactose, a strain of *Saccharomyces cerevisiae* shows greater oxidative activity, an essentially unchanged anaerobic fermentation rate and an increase in total growth, compared with the unadapted yeast grown on the same medium but containing instead 2% glucose as major carbon source. The galactose-grown yeast possesses a generally higher level of oxidative metabolism, with enhanced $-Q_{O_2}$ values (up to 500%) for glucose and other oxidizable substrates, a higher content of cytochrome components and a much higher cytochrome oxidase activity.

With lower concentrations of sugar in the growth medium, the oxidative activities of glucose-grown cells approach those of galactose-grown cells; the results suggest that glucose, when present in greater than a very low optimal concentration (0·05% or less), inhibits the formation of oxidative enzymes. Mannose or fructose (which are readily metabolized by the yeast without need for adaptation) or a mixture of glucose and galactose produce low activities similar to those given by glucose.

The magnitude of the differences between glucose-grown and galactose-grown yeasts is greatly influenced by the composition of the basal growth medium. The most marked differences have been obtained with a casein digest-marmite medium; with richer media the differences are lessened, and when grown on a complex malt-yeast extract-inorganic salts medium the glucose-grown and galactose-grown yeasts are practically indistinguishable with respect to oxidative capacities and total growth.

Production of Indole Metabolites by Yeast. By A. A. EDDY (*Brewing Industry Research Foundation, Redhill*)

Cultures of a strain (NCYC 77) of *Saccharomyces cerevisiae* were grown on a glucose-ammonium sulphate synthetic medium containing vitamins and mineral salts and supplemented with DL-tyrosine, or L-tyrosine, or DL-phenylalanine at a concentration of 300 μg./ml. By the methods of paper chromatography they were shown to contain small amounts (*c.* 1 μg./ml.) of indole and tryptophol, together with an as yet unidentified neutral indole derivative. The latter was detected in greater amount (*a*) when indole had been added to the growth medium, (*b*) when the normal complement of pyridoxin had been omitted as well as the supplement, and (*c*) in cultures of a mutant yeast with a specific requirement for tryptophan for growth.

Followed by a Symposium on

MECHANISMS OF MICROBIAL PATHOGENICITY

The Meaning of Pathogenicity. By A. A. Miles (*Lister Institute, London*)

The Role of Toxins in Pathology. By W. W. Van Heyningen (*Sir William Dunn School of Pathology, University of Oxford*)

The Pathogenesis of Diphtheria. By A. M. Pappenheimer (*University College of Medicine, New York*)

On the Biochemical Mechanism of Action of Gas-gangrene Toxins. By M. G. Macfarlane (*Lister Institute, London*)

Botulinum and Tetanus Toxins. By G. Payling Wright (*Guy's Hospital Medical School, London*)

Properties and Structures of Tubercle Bacilli concerned in their Pathogenicity. By R. J. Dubos (*Rockefeller Institute, New York*)

Studies on the Chemical Basis of the Pathogenicity of *Bacillus anthracis* using Organisms Grown *in vivo*. By H. Smith and J. Keppie (*Microbiological Research Department, Porton*)

Glutamyl Polypeptide Synthesis and Virulence of *Bacillus anthracis*. By R. D. Housewright (*Camp Detrick, Frederick, U.S.A.*)

The Basis of Virulence for Mice of *Pasteurella pestis*. By T. W. Burrows (*Microbiological Research Department, Porton*)

The Pathogenicity of Protozoal and other Parasites; General Considerations. By F. Hawking (*National Institute for Medical Research, London*)

The Comparative Pathogenicity of Protozoa in their Vertebrate and Invertebrate Hosts. By P. C. C. Garnham (*London School of Hygiene and Tropical Medicine*)

The Pathogenicity of Plasmodia and Entamoebae. By B. G. Maegraith (*Liverpool School of Tropical Medicine*)

Host-parasite Relations and Pathogenesis in Infections with *Entamoeba histolytica*. By C. A. Hoare and R. A. Neale (*Wellcome Laboratories of Tropical Medicine, London*)

Pathogenicity of Fungi in Man and Animals. By G. C. Ainsworth (*University College, Exeter*)

Pectic Enzymes secreted by Pathogens and their Role in Plant Infection. By R. K. S. Wood (*Imperial College, London*)

The Role of Toxins in the Aetiology of Plant Diseases caused by Fungi and Bacteria. By P. W. Brian (*Akers Research Laboratories, Welwyn*)

Studies on the Mechanism of Invasion of Plants by Vascular Pathogens. By W. G. Keyworth (*National Vegetable Research Station, Wellesbourne*)

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